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W. J. CROZIER JOHN H. NORTHROP W. J. V. OSTERHOUT

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*No. 1. SIMON FLEXNER, J. W. JOBLING, and MAUD L. MENTEN. Experimental studies on tumor. Plates 1–28. (Issued June 30, 1910.) Price, $1.00.

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A THEORETICAL CONSIDERATION OF THE ACTION OF X-RAYS ON THE PROTOZOAN COLPIDIUM COLPODA.

By HARRY CLARK.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Accepted for publication, February 7, 1927.)

I. INTRODUCTION.

To show that the variation in reaction among the individuals of a group of apparently similar organisms fits a probability curve is, of course, not an end in itself, but rather a basis of conjecture as to the mechanism which provides the random element without which the theory of probability is meaningless. It is generally assumed that the reactions of various individuals differ because the individuals themselves are different. It is interesting, however, to consider the possibility that in some cases, where the organism is relatively simple, the individuals are essentially similar and the random element is inherent in the agent or in its primary effects. For example, if the agent is radiation, the quite generally accepted theory of absorption in *quanta* provides the necessary random element; and it may be that this is sufficient to account for the variations which we observe in the reaction to the rays of apparently similar simple organisms.

In his recent work on the action of x-rays on *Colpidium colpoda*, Crowther[1] finds that if he plots the number of survivors against the dose of radiation administered the result is a sigmoid curve; and, assuming the animals to be essentially alike, he shows that this is the kind of curve to be expected if the animal dies as a result of a certain number, n, of discreet events or "hits," all equally effective, and if the probability per unit dose of making a hit is constant. He finds, further, that theory agrees quantitatively with experiment when λ_e, the probability per e-unit of dose, is 5.9×10^{-4}, and n (for immediate death) is 49.

To account for this very small value of λ_e, he makes use of an inter-

[1] Crowther, J. A., *Proc. Roy. Soc. London, Series B*, 1926, c, 390.

esting hypothesis, which he advanced some time ago[2] for a similar purpose, that the effect is confined to some very small body or structure inside the animal. In doing so, he introduces certain difficulties, in my opinion. No general objection is raised against this hypothesis, which we may refer to as the "small-body theory." In this particular case, however, it appears to be inconsistent with the phenomenon which it was devised to explain, unless it is supplemented by certain rather novel assumptions as to the nature of the destructive effect of radiation on tissue. The nature of these assumptions will be brought out in what follows. An alternative explanation of the small value of λ_v which does not involve the small-body theory will also be suggested.

II. Primary and Secondary Effects of X-Rays.

The primary effect of x-rays on the light atoms of which living matter chiefly consists is the ejection of a high speed electron from some of them chosen at random in both space and time. Each of these primary electrons ionizes a large number of other atoms by collision before it comes to rest. This secondary ionization appears to be the only effect which we need consider. There is no good reason for supposing that the atom from which a primary electron has been ejected is the seat of any considerable part of the destructive effect; the disintegration of the particular molecule which contains this atom can scarcely be thought of as being more important, in general, than that of any other molecule. There is, on the other hand, plenty of evidence in favor of the view that the destructive effect is associated with the ionization produced by the high speed electron, in comparison with which the ionization by direct absorption of the rays is negligible. In very simple photochemical systems, the reactions produced by x-rays or by α- or β-rays proceed at a rate which is directly proportional to the rate of ionization; it would be rash, of course, to assert that this is true in the case of tissue destruction.

If the x-rays are monochromatic, the primary electrons are all ejected with the same speed and energy; they travel approximately equal distances before stopping, and they knock off about the same

[2] Crowther, J. A., *Proc. Roy. Soc. London, Series B*, 1924, xcvi, 207.

number of secondary electrons. The various quantum events or units are, therefore, much alike. Likewise, the events which consist in the production of the various secondary electrons are alike in one respect at least—all of the electrons have the same properties.

Doubtless one or the other of these units corresponds to the hit mentioned above. In what follows, I shall refer to the release of a secondary electron as an *electron-hit*, and to the emission of a primary electron wth its attendant phenomena as a *quantum-hit*. The units of destructive effect dealt with in Crowther's analysis will be called *effective hits*.

III. Some Quantitative Estimates.

Crowther used the K radiation of molybdenum, the α lines of which have a mean frequency of 4.23×10^{18} per second. The primary electron is thus ejected with an amount of energy, $h\nu$, equal to 2.76×10^{-8} ergs. Dividing this by 5.5×10^{-11} ergs, the work required, on the average, to release a secondary electron in air according to Rutherford,[3] we find that each primary electron releases about 500 secondary electrons. This number will be denoted by E_q.

The fourth power law, together with Whiddington's[4] constant for air, shows that the maximum path length of the primary electron in air must be about .2 cm. The value taken directly from one of Sadler's[5] curves is .22 cm. Evidently the law holds nicely even for these very soft rays. In tissue, assumed equivalent to air of unit density, the maximum path, L, is, therefore, about 2.6×10^{-4} cm.

The paths of the primary electrons are, in general, not straight, and, in consequence, the distance in a straight line from the beginning to the end of the path is generally less than L. Consider a plane layer of air, the thickness of which, x, is uniform and somewhat less than L. If a great number of electrons enter this layer through one of its faces, all with the same speed, but in all possible directions, some of them will emerge from the opposite face with some part of their original energy. A fraction, then, of the energy which goes into the layer on one side comes out on the other side. Sadler[5] has shown that

[3] Rutherford, E., Radioactive substances and their radiations, Cambridge, 1913, 159.

[4] Whiddington, R., *Proc. Roy. Soc. London, Series A*, 1911–12, lxxxvi, 360.

[5] Sadler, C. A., *Phil. Mag., Series 6*, 1910, xix, 337.

the value of this fraction is given by $e^{-\mu s}$ in which μ is the mass-"absorption" coefficient of air for electrons of a particular initial speed. For molybdenum K electrons, he gives 1.18×10^4 as the value of μ. μ does not depend to any great extent on the nature of the absorbing material; we may say with safety that it has the same value for air and for tissue. This is the assumption on which we have already computed L.

The exponential law is, of course, not strictly true; it is inconsistent with the existence of a maximum path length. When the absorbing material reaches a thickness such that only those electrons which have travelled very nearly in a straight line can get through, a slight increase in the thickness will stop them all.

The distribution of secondary electrons along the path of the primary electron must now be considered. In any small part of the path, they are distributed very nearly at random—as nearly as the fine structure of matter permits. The space rate of ionization increases, however, as the velocity decreases. At the end of the path, the ionization is probably very intense. Glasson[6] states that over a considerable part of the path, at least, this rate (the number of electrons per cm.) varies inversely as the square of the velocity of the primary electron. This law may, of course, be derived directly from the fourth power law. On this basis, a simple calculation, which need not be given here, shows that by the time the primary electron which we are considering has reached the middle point of its path, it has released 150 secondary electrons, and that, at this point, the mean distance between consecutive electrons is about 7.3×10^{-7} cm. This is enough to show that we are not to think of the secondary ionization as being almost wholly confined to a small region near the end of the path.

Friedrich's e-unit of radiation is the amount required to release in 1 cc. of air at N.T.P. 1 electrostatic unit of charge of either sign, or 2.1×10^9 electrons. The mass absorption coefficient of tissue is the same as that of air and it is reasonable to assume, as Crowther does in his earlier paper, that the number of secondary electrons *per quantum* is the same. Since the tissue with which we are dealing is approxi-

[6] Glasson, J. L., *Phil. Mag., Series 6*, 1910, xxii, 647.

mately of unit density, an e dose corresponds to the production of 1.63×10^{12} secondary electrons per cc. of tissue. This number will be called E_e.

IV. The Small-Body Theory.

That λ_e has been found to be very small shows that very few of the hits received by the animal, whether electron-hits or quantum-hits, are effective. From the fact that the atoms from which the high speed electrons are ejected are distributed at random in space, it follows that the probability that a high speed electron will be ejected from within any small portion of the animal is directly proportional to the volume of the portion considered and independent of its position. The same is true of the probability that a secondary electron will be released within the portion considered, provided, of course, that the volume is such that the electron-hits occur independently of one another. Assuming that the destructive effects are confined to some small body within the animal, we may assign volumes to this body such that either of these probabilities will assume any desired value,—in particular the value 5.9×10^{-4} in which case every hit within the small-body will be effective. On the hypothesis that the electron-hit corresponds to the unit of destructive effect, the diameter of the body (assumed approximately spherical) must be about 8.8×10^{-6} cm. Similarly on the quantum-hit hypothesis, the diameter is about 7.0×10^{-5} cm.[7]

Let us now inquire whether this theory is consistent with the postulates on which the statistical treatment of the problem is based. The postulates are: (1) that all effective hits are equally effective, and (2) that λ_e is constant.

Let us consider first the bearing of the electron-hit hypothesis on the small-body theory. L, the path length of the primary electron, is 29.4 times the diameter of the small-body appropriate to the hypothesis that the individual secondary electron corresponds to a unit of destructive effect. Since 500 such electrons are released by the primary electron in traveling a distance equal to L, it is evident that

[7] As the result of an error in calculation, which Dr. Crowther discovered after publication, the diameters assigned to the body in his paper differ somewhat from those given above.

in most cases, in which a high speed electron traverses the small body, it will release more than one secondary electron inside it. Those events, then, which are at random in time, are not the electron-hits at all, but rather showers of electron-hits, and the number of electrons per shower must vary within very wide limits, because the intensities of ionization, at the beginning and the end of the path, respectively, differ so much, and because the length of the path through the body varies from zero to the length of the diameter,—even more if the path is not straight. The average number of electrons per shower is, of course, very great in the case of primary electrons which enter from outside and come to rest inside the body; and correspondingly small for those which are ejected from within the body. For primary electrons which pass through the body, the average number of secondaries per shower is about 11, since the mean length of a great number of straight paths through a sphere, chosen at random, is equal to two-thirds of the diameter. Electron-hits are not then at random in time—not even approximately so—and postulate 2 is not fulfilled.

It is apparent then that we must abandon either the electron-hit idea or the small-body theory.

We have now to deal with the quantum-hit hypothesis in its relation to the small-body theory. Let us assume for the sake of the argument that the distribution of the destructive effect along the path of the primary electron is the same as that of the secondary ionization—which would be true if we were dealing with a simple photochemical system. It is evident that some of the primary electrons, ejected from atoms inside the small-body, must escape from the body with a considerable part of their initial energies. Likewise, other high speed electrons, ejected from matter outside the small-body, will enter it before coming to rest. In these cases, the effectiveness of the hit will be less than in the cases in which the whole path lies inside the body. Hits of this kind will be referred to in what follows as "partial" hits.

The relative number of partial hits cannot be so small as to be negligible. On the quantum-hit hypothesis, the diameter of the small-body is 7.0×10^{-5} cm., whereas L is 2.6×10^{-4} cm., *i.e.* 3.7 times the diameter. In order to make a very rough estimate of the relative importance of the partial hits, we set x equal to 3.5×10^{-5} cm., the

radius of the sphere, in the expression $e^{-\mu x}$ discussed in Section III, and we find that, of the energy associated with high speed electrons ejected from points midway between the faces of a layer of tissue of thickness equal to the diameter of the small-body, 66 per cent escapes from the layer. If we say that 66 per cent of the electrons escape, we shall make an underestimate, for each of the escaping electrons has lost a part of its initial energy. If we say that 66 per cent of the high speed electrons, ejected from the center of the small-body, escape, we shall underestimate the number still further, because the radius of the sphere is much smaller than the mean of the distances between a point in the middle of the plane layer and the points where the electrons escape from the surfaces of the layer.

Of the high speed electrons ejected from the center of the small-body, then, at least 66 per cent escape; of those ejected from points near the surface of the body, at least 50 per cent escape. Let us say that at least 50 per cent of all high speed electrons released within the small-body escape from it. Now for every one which escapes, another enters from outside. The whole number of hits, both total and partial, *i.e.* the whole number of those events which occur at random in time, is then increased by 50 per cent and two-thirds of them are partial hits. To keep the whole number down to 49, the body must be made smaller, and this will make the relative number of partial hits still greater. From what has been said in Section III about the distribution of ionization along the path it appears that we must give up either the small-body theory or the idea *that the distribution of the destructive effect along the path of the high speed electron is similar to that of the ionization.*

It is conceivable that the destructive effect, though brought about by ionization, is not measured by it; that it is conditioned in some way by the density of ionization or otherwise. It might be supposed, for example, that at the end of a path a small portion of tissue is injured so seriously that repairs are impossible; that at other points along the path the injury, being diffuse, is rapidly made good. If this were true and if the permanent injury which corresponds to an effective hit were confined to a very small region—to 1 per cent, let us say, of the path length, no objection could be raised against the small-body theory.

This idea, that the hit is localized in some very small part of the path, is the assumption referred to in the introduction. It should be noted that it amounts to something more than the assumption that some single molecule, peculiarly essential to the organism, happens to lie in the path of the primary electron and to be destroyed by it; if this were the case, the introduction of the small-body theory would no longer explain why all quantum-hits are effective and equally effective. Whether or not the difficulty of reconciling this assumption with the known facts of photochemistry is more than sufficient to compensate for the usefulness of the small-body theory is a matter of personal judgement.

V. An Alternative Hypothesis.

If the small-body were subdivided into a great number of much smaller bodies, and if these smaller bodies were placed as far apart as possible, the probability that a quantum would make two or more effective electron-hits would be made smaller. To make it negligible, however, the bodies would have to be very small in comparison with the mean distance between consecutive electrons in a shower. This suggests that an effective hit may correspond in some way with the destruction of molecules of a certain kind or kinds distributed throughout some considerable part of the tissue. The number, N, of such molecules present in the animal at the beginning of an exposure would have to be very great, of course, in comparison with n, which is 49, otherwise λ_e would become appreciably smaller as more and more effective hits were made. That the loss of so small a fraction of these molecules should have so profound an effect suggests either that they are essential parts of some structure or that the destruction of the molecule is followed by a recombination of the component atoms to form a molecule of a new substance which is highly toxic. The postulate that all effective hits must be equally effective seems to favor this latter idea, and to require that we restrict ourselves to one kind of molecule. We shall consider, then, that the making of a molecule of the toxic substance Y constitutes an effective hit. It appears highly improbable that the destruction of a molecule of substance X would always result in the production of a molecule of Y; it would certainly be more reasonable to suppose that Y is formed only when X loses

certain particular electrons. The problem cannot be analyzed, of course; too little is known about the fine structure of matter. In what follows, an attempt will be made to estimate the various quantities involved in the relatively simple case where Y is formed when X, represented by an idealized molecule, loses one particular electron. It will be assumed that in a microscopic sense the molecules of X are at all times distributed at random in space insofar as the finite size of the molecule permits,—the arrangement to be expected in a solution. It will appear further on that the molecule would have to be extremely large to have an appreciable effect on this distribution; it will be assumed tentatively that it has none.

Multiple Effective Hits Made by One Quantum.

Let us assume for the moment that, in the ordinary sense, X is distributed uniformly throughout the whole volume of the animal. If the probability that a quantum, falling entirely inside the animal, will make an effective hit be represented by p; and if V be the volume of the animal, then

$$p = \frac{\lambda_e E_q}{E_e V}. \tag{1}$$

V may be taken as 10^{-7} cc. Using the value of λ_e given by Crowther, and the values of E_q and E_e found in Section III, we find that $p = 1.8 \times 10^{-6}$. Now it is not the quantum as a whole, but rather the individual secondary electrons which correspond to the hits. In the language of probability we may, therefore, speak of the number of "trials" per quantum. If the molecule of X were so very small that it would never lose two or more electrons, the number of trials would be equal to E_q. If the molecule were larger, the number of trials would be less than E_q. When p is less than 1, a decrease in the number of trials, corresponds to a decrease in the ratio of p_r to p_1; p_r being the probability that the quantum will make exactly r effective hits and p_1 the probability of exactly one such hit. For example, if the number of trials were 1, the probability of a multiple hit would be absolutely zero. To find the maximum value of this ratio, which we may call R_r, we take the number of trials as infinite in which case R_r is $p^{r-1}/r!$ In particular, R_2 is $p/2$ or 9.0×10^{-7}.

R_r is so small that we may now reconsider the assumption that X is distributed uniformly throughout the whole volume of the animal. Other things being the same, p is proportional to the number of molecules of X per unit volume in the region where the quantum falls. If in some part of the animal the concentration of X were 1000 times as great as the mean concentration, then in this part p would be 1.8×10^{-3}; only about one effective hit in a million would be a "double," and one in less than 10^{12} a "triple" hit. p, for a particular quantum, cannot, of course, be greater than the value corresponding to the maximum concentration which the primary electron encounters; the concentration may change from point to point, therefore, as abruptly as desired.

It is evident, then, that effective hits are at random in time and that the molecules of X to be hit effectively are chosen at random, even though no unreasonable restrictions are placed on the way in which X is distributed.

The Size of the Molecule.

We have now to deal with the slow change in λ_e which takes place in consequence of the fact that N is finite. Let P_1 be the probability that a destroyed molecule of X, chosen at random from among the whole number of those that have been hit, will have lost exactly one electron; and let P' be the probability that a molecule, chosen at random from among all those which have lost exactly one electron, will have lost the particular electron required. In the normal case, the making of n effective hits corresponds to the destruction of n/P_1P' molecules of X, and, therefore, n/NP_1P' represents the relative change in λ_e. This latter quantity must then be small; just how small is a matter of judgment. It ought certainly to be smaller than the errors in experiment, and the results of Crowther's experiment fit the theoretical curve very nicely. The values of P_1 and P' depend on the properties of the molecule of X. As the volume, v, and the complexity of the molecule increase, both P_1 and P' diminish; furthermore, since Nv may not be greater than the whole volume of the animal, the maximum value which we may assign to N diminishes. The hypothesis is, therefore, consistent for a given value of v provided the corresponding value of NP_1P' is sufficiently large in comparison with

n and provided the value of P_1P' appropriate to a molecule of volume v is not so small that N has to be greater than V/v. It is obvious that these conditions are more easily fulfilled the smaller and simpler the molecule. We have to find out, if possible, whether or not they are fulfilled when the molecule is fairly large.

In order to estimate P_1 and P', it is necessary to make certain idealizing assumptions as to the nature of the molecule and to assign a definite size to it. To make it possible to treat P_1 statistically, it is assumed that the molecule will behave as though its electrons were distributed at random inside a sphere, the volume of which is the same as that of the molecule; the probability of releasing an electron being the same for all of them. For convenience, the diameter of the sphere is set equal to 10^{-7} cm. The volume is then equal to that of the molecule of oleic acid according to Langmuir.[8]

In the case of a complex organic molecule the electrons must be fairly evenly distributed throughout what we call its volume, *i.e.* the room which it occupies when stacked with other molecules to constitute matter in the solid state. Such a distribution, together with the movements of the electrons, and the random orientation of the molecule with respect to the path of the high speed electron may reasonably be thought of as equivalent to a random distribution. The probability that an electron will be released from the molecule is then directly proportional to the path length through the molecule. The constant of proportionality will be nearly enough equal to that for tissue in general, if we assign to the molecule the same number of electrons as that in the molecule of oleic acid, *i.e.* 158. P' will then be .0063. It will appear presently that, for a molecule of this size, the conditions imposed by the size of the animal and the desired constancy of λ_s are fulfilled with a margin of safety which is so great that the errors involved in idealizing the molecule need not be small.

We must now try to estimate P_1. Since the particular electron to be removed may be anywhere, we must suppose that it is in the worst place, *i.e.* at the center of the sphere. It will have the same chance of being hit wherever it is, but if it is at the center, the primary electron must traverse the longest path through the sphere to reach it,

[8] Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1848.

and the probability of removing two or more electrons from the same molecule increases with the path length. It has been shown in Section III that, on the average, 30 per cent of the ionization, *i.e.* 150 secondary electrons, lie in the first half of the path of the high speed electron, and that even at the midpoint of the path, consecutive secondary electrons are no closer together on the average than 7.3×10^{-7} cm., which is over seven times the maximum path length through our molecule. Let us confine our attention to the first half of the path for the moment. If the high speed electron were shot into a solid mass of X, the molecules being lined up in such a way that it would traverse a diameter of each, only about one molecule in seven at the midpoint of the path would lose an electron. When we remember that electrons are released farther and farther apart as we go from the midpoint toward the beginning of the path, we see that the number of cases in which a molecule loses two or more electrons must be very small in comparison with the number of those in which it loses only one. We shall make no great error if we assume that all hits in the first half of the path are "single hits." There are, undoubtedly, many single hits in the last half of the path, where the ionization is more intense, but we shall ignore them in order to make sure that we are not over-estimating P_1. The total number of single hits is then equal to 150, the number of electrons in the first half. It should be remembered that we assumed that the path follows the diameter of the molecule for the purpose of estimating the relative number of double hits. The number of single hits just found, 150, has nothing to do with the exact location of the path.

Now P_1 is, in the long run, the ratio of the number of molecules which have lost one electron to the total number destroyed by the loss of any number of electrons. For the average quantum, falling in a mass of X in the pure state, the whole number of molecules destroyed, which we will denote by M, must be less than 500, for some of the molecules lose two or more electrons. If all of the electrons in the last half of the path were lost by the same molecule, an absurd assumption, M would be 151, and P_1 would be 1. If all of the hits in the second half of the path were doubles, M would be 325 and P_1 would be .46. This is the minimum value of P_1. To sum up—P_1 lies somewhere between .46 and 1, and M lies between 150 and 500. Even

though the value of P_1 depends in part on M, we must consider the limiting values separately.

λ_e, the probability per e-unit of making an effective hit, is given by

$$\lambda_e = \frac{E_e}{E_q} \cdot N v M P_1 P'. \tag{2}$$

Substituting the limiting values of M, found above, we see that NP_1P' must lie between 6.9×10^5 and 2.3×10^6. To be on the safe side, we use the smaller of these numbers to test the constancy of λ_e. $n \div NP_1P' = 7.1 \times 10^{-5}$. In the normal case, λ_e may change, then, by as much as .007 of 1 per cent. Such a change is too small to consider.

Now we consider the maximum value of N. To make N as large as possible, we divide the greater value of NP_1P', which is 2.3×10^6, by the minimum value of P_1P', which is $.46 \times 6.3 \times 10^{-3}$, and N comes out to be 8.0×10^8. The total volume of X in the animal is then 4.2×10^{-13} cc., which is only 4.2×10^{-6} times the volume of the animal. The "volume" concentration of X is then only .0004 of 1 per cent; it is, of course, so small that cases will be very rare in which the finite size of the molecule interferes with the assumed random distribution.

The margins of safety in the variation of λ_e and in the total volume of substance X are obviously so great that the error involved in assuming that the real molecule behaves like the ideal one may also be very great without rendering the general hypothesis untenable. There can be little doubt that, if an effective hit corresponds to the removal of a particular electron from a molecule of X, the molecule of X may be fairly large and complex.

VI. CONCLUSION.

If we accept the idea that the reactions of living matter to x-rays are the result of ionization, we find that Crowther's small-body theory serves to explain the small value of λ_e only provided it be assumed further that the unit of destructive effect which corresponds to an effective hit is associated with the quantum and that it is localized in a region the dimensions of which are very small in comparison with the path length of the high speed electron.

It is suggested that there exists in the animal a substance, X, distributed throughout a considerable part of the tissue, a molecule of which turns into a molecule of a substance Y when it loses a particular electron, and that the formation of a molecule of substance Y constitutes an effective hit. This hypothesis seems to be consistent if the molecule of X is not too large. There is, of course, no good reason for supposing that it is the true explanation of the phenomenon. It is put forward merely to show that we may accept the theory that the variations in reaction are inherent in the x-ray itself without accepting the small-body theory.

VII. SUMMARY.

1. The theory which Crowther has advanced to account for the variation of the lethal dose of roentgen rays among the individuals of a group of *Colpidium colpoda* is reviewed.

2. It is shown that the use of his small-body theory to explain the small value of λ_e leads to certain further assumptions about the nature of the destructive effect.

3. An alternative hypothesis is discussed.

TIME RELATIONS OF GROWTH.

III. Growth Constants during the Self-Accelerating Phase of Growth.*

By SAMUEL BRODY.

(From the Department of Dairy Husbandry, University of Missouri, Columbia.)

(Accepted for publication, January 31, 1927.)

I.

INTRODUCTION.

The period of growth of multicellular organisms, and of populations of organisms, may be divided into two fairly distinct phases: (1) a *self-accelerating phase* during which the time rate of growth increases with the increase in size of the organism or the population; and (2) a *self-inhibiting phase* during which the time rate of growth decreases with the increase in size of the organism, or population. The question of mechanisms bringing about the general similarity in the course of growth of animals, plants, and populations, need not be gone into in this place except to note that the course of growth is in all these cases governed, directly or indirectly, by the same two primal forces: (1) the force inherent in all organisms to reproduce at a constant percentage rate; and (2) the growth-inhibiting forces resulting from the finite nature of the universe in which the organisms find themselves.

The purpose of this series of papers is merely to present quantitative analyses of growth curves, with special reference to developing methods for computing *rational* growth constants. The first two papers[1] were concerned with the *self-inhibiting phase* of growth, that is the phase of growth following the major inflection in the time curve

* The principal portions of this paper have been presented before the Physiological Section of the Botanical Society of America, in Philadelphia, December 30, 1926.

[1] Brody, S., *J. Gen. Physiol.*, 1925-27, viii, 233. Brody, S., Sparrow, C. D., and Kibler, H. H., *J. Gen. Physiol.*, 1925-26, ix, 285.

of growth. This paper is concerned with the *self-accelerating phase* of growth.

II.

Proposed Methods for Computing Growth Rates.

Four methods have been proposed for computing growth rates.

1. The Method of Minot.—Minot computed the rate of growth by dividing the gain in weight during a finite unit of time by the weight at the beginning of the unit of time, as represented by the equation

$$R = \frac{W_2 - W_1}{W_1} \tag{1}$$

or

$$W_2 - W_1 = R\,W_1 \tag{1a}$$

in which W_1 and W_2 are, respectively, the weights at the beginning and the end of the unit of time, R is the relative (or when multiplied by 100, the percentage) rate of growth.

Employing this method he was led to the conclusion that, in warm blooded animals, the percentage rate of growth declines from 1000 per cent per day shortly after fertilization, to 3 to 7 per cent per day at the time of birth or hatching.

There is this objection against the use of equation (1), for the self-accelerating phase of growth: It is based on the assumption that growth is a discontinuous process; *i.e.*, that the increments are added at arbitrary time intervals, t_1, t_2, t_3. . . As a matter of fact, statistically considered, growth is a continuous process, and the relative rate of growth, must, therefore, be represented by the equation

$$k = \frac{dW/dt}{W} \tag{2}$$

or

$$\frac{dW}{dt} = kW \tag{2a}$$

in which k is the instantaneous relative rate (or when multiplied by 100, percentage rate) of growth, corresponding to R in equation (1).

The error introduced by the use of equation (1) is very considerable, as may be seen from the following considerations.

From equation (2), at time t_1, the weight, W_1, of the organism is represented by the equation

$$W_1 = Ae^{kt_1} \tag{3}$$

At time t_2, it is represented by

$$W_2 = Ae^{kt_2} \tag{3a}$$

Subtracting the former from the latter we obtain,

$$W_2 - W_1 = Ae^{k(t_2 - t_1)}$$

Dividing by (3),

$$\frac{W_2 - W_1}{W_1} = e^{k(t_2 - t_1)} - 1$$

Transposing and taking logarithms,

$$ln\left(\frac{W_2 - W_1}{W_1} + 1\right) = k\,(t_2 - t_1)$$

For 1 unit of time,

$$k = ln\left(\frac{W_2 - W_1}{W_1} + 1\right) = ln\,(R + 1) \tag{4}$$

Numerical relations between k, the instantaneous rate of growth, and R, the rate of growth as determined by Minot, may be computed by substituting the values of R in equation (4). The results for a series of substitutions are presented graphically in Fig. 1. Fig. 1 makes it clear that Minot's method (equation (1)) cannot be used for computing the relative rate of growth when the rate exceeds 10 per cent for the unit of time under consideration.

2. The Method of Pearl.—In 1907

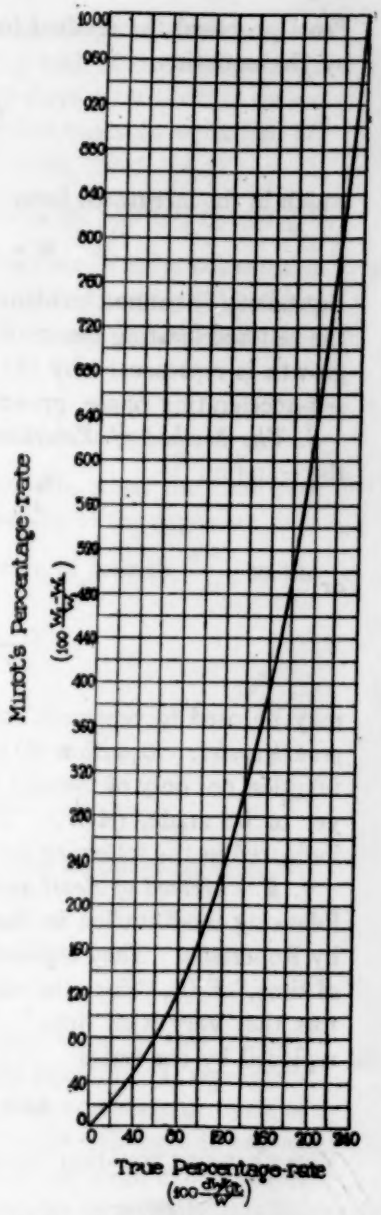

FIG. 1. The relation between the increase in percentage rate as computed by the logarithmic method and by the arithmetical method of Minot.

Pearl proposed the method for computing growth rates represented by the equation

$$\frac{dW}{dt} = \frac{k}{t - a} \tag{5}$$

which in the integrated form is,

$$W = A + k \ln (t - a) \tag{5a}$$

Equation (5) cannot, evidently, be used to represent growth during the self-accelerating phase of growth, inasmuch as the time rate of growth is represented by (5) to decline with time, while during the self-accelerating phase, growth increases with time.

3. The Method of Robertson.—In 1908 Robertson suggested that the equation

$$\frac{dW}{dt} = k\, W\, (A - W) \tag{6}$$

or

$$\frac{dW/dt}{W\,(A - W)} = k \tag{6a}$$

may be used to represent certain phases of growth termed by him *growth cycles*. Equation (6) indicates that the velocity of growth is a function not only of the size, W, of the organism, but also of growth yet to be made, $(A\text{-}W)$. The objections against this equation are indicated in the following sections.

4. The Method of Pearl and Reed.—Pearl and Reed introduced the following modification in the autocatalytic equation (6), employed by Robertson. They replaced k, by "some as yet undefined function of time," $F\,(t)$, "since the rate of growth of W is dependent upon factors that vary with time." They then assumed that $F\,(t)$ may be replaced by the series

$$k_1 t + k_2 t^2 + \ldots + k_n t^n$$

thus changing equation (6) into

$$\frac{dW/dt}{W(A - W)} = F\,(t) = k_1 t + k_2 t^2 + k_3 t^3 + \ldots + k_n t^n \tag{7}$$

They found the integrated form of equation (7) to be elastic enough to fit the growth curve of the rat beginning with 10 days after birth.

Since, however, the period preceding 10 days after birth is an exceedingly important one, and since the constants in equation (7) do not have definite physical meaning (thus, when $(A\text{-}W) = 1$, $\frac{dW/dt}{W} = k_1 t + k_2 t^2 + \ldots + k_n t^n$; what is the physical meaning of $k_1 t, k_2 t^2 \ldots$?) therefore the method of Pearl and Reed is not suitable for the purpose under consideration (which is to evaluate *rational* growth constants; *i.e.*, constants having well defined physical meaning).

III.

The Method Employed in This Paper.

During the self-accelerating phase of growth, when the time rate of growth increases with the increase in the size of the organism, it is reasonable to attempt to relate the time rate of growth, $\frac{dW}{dt}$, to the size, W, of the organism, by the function

$$\frac{dW/dt}{W} = k \tag{2}$$

or

$$\frac{dW}{dt} = k\,W \tag{2a}$$

The first thought is that the constancy of k may be tested by integrating (2) and solving for k

$$k = \frac{ln\ W_2 - ln\ W_1}{t_2 - t_1}$$

As a matter of fact this is an impractical procedure for two reasons. First, it is not known for how long a period equation (2) represents the data; *i.e.*, $t_2 - t_1$ may represent more than one stage or cycle of growth; second, ratios are very sensitive to slight changes in one or both of the variables. This fact taken with the large experimental errors involved in this work, makes the results apparently erratic.

A better method is to plot the logarithms of the size, or weight, of

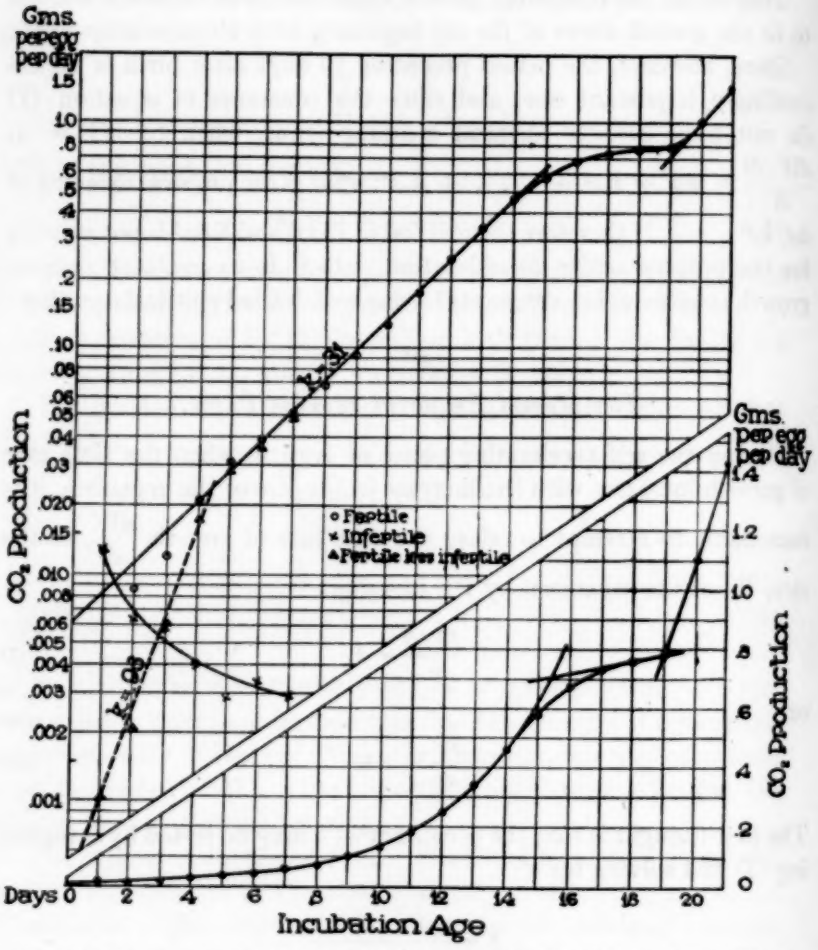

FIG. 2. The course of carbon dioxide excretion in the chick embryo with advancing age plotted from data by Atwood and Weakley. From 0 to 4 days, the instantaneous percentage rate of growth appears to be 98 per cent per day (the amount of carbon dioxide excretion is doubled once in .7 day, or once in 17 hours); between 4 and 14 days, the rate of increase in carbon dioxide excretion is 31 per cent per day (it is doubled once in 2.2 days). The pause in the curve coincides with the maximum in the mortality curve (of Fig. 3), and with the change in the mode of respiration (see text).

the organism, against age, since the integrated form of (2) may be written

$$W = Ae^{kt}$$

Therefore

$$\ln W = \ln A + kt$$

If the data points of the logarithms of weights plotted against age are distributed about a straight line, then the percentage rate of

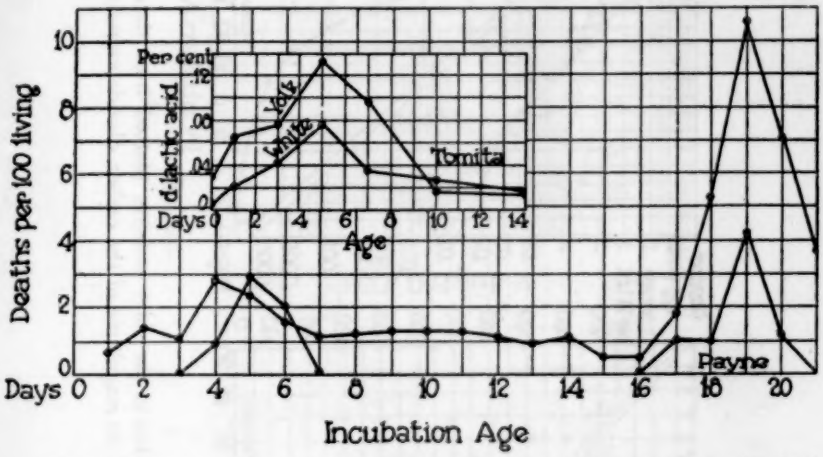

FIG. 3. Age changes in the percentage mortality with increasing age in the chick embryo. Open circles represent mortality data of embryos incubated in an incubator; solid circles represent incubation under hens. The first peak in the mortality curve corresponds with the peak in the concentration of lactic acid as found by Tomita. The second peak in the mortality curve coincides approximately with the pause in the growth curves (Figs. 2 and 5).

growth, represented by 100 k, is constant, and k is the growth constant we are seeking. Instead of plotting logarithms of weights, we may plot the data on paper on which the axis of ordinates is divided logarithmically (*i.e.* on arithlog paper).

IV.

The Results of Plotting Growth Data on Arithlog Paper.

Fig. 2 represents the course of increase in carbon dioxide excretion with age in the chick embryo, as plotted on arithlog paper. The rate

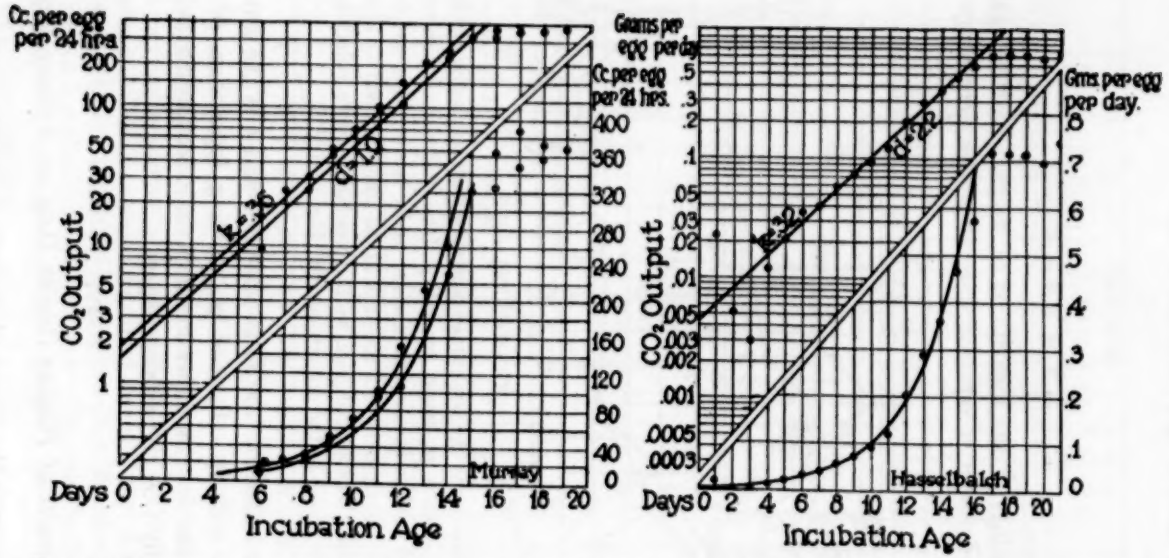

FIG. 4. The course of carbon dioxide excretion in the chick embryo, plotted from data by Hasselbalch and Murray. *d* refers to the time in days in which the magnitude of the carbon dioxide excretion is doubled.

of growth, as measured by the increase in carbon dioxide production, is constant between the 1st and 4th day of incubation. The increase is of the order of 100 per cent per day, and not 1000 per cent as postulated by Minot. From the 4th to the 15th day, the increase is likewise constant; it is 31 per cent per day.

The pause in the curve between 17 and 19 days, is, no doubt, associated with the change in the mode of respiration (from the aquatic

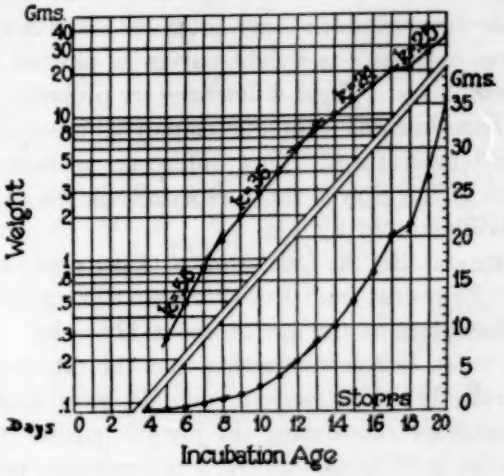

FIG. 5. Growth in wet weight of the chick embryos plotted from data by Lamson and Edmond (Storrs Agricultural Experiment Station). The curve appears to consist of four segments each of which represents growth at a constant percentage rate. The pause in the curve between 17 and 18 days coincides approximately with the second peak in the mortality curve (Fig. 3). Growth at an instantaneous rate of 56 per cent per day indicates that the body weight is doubled once in 12 days; at 36 per cent per day, the body weight is doubled once in 1.9 days; at 24 per cent per day, the body weight is doubled once in 29 days.

to the terrestrial mode) which takes place at this time. Fig. 3, representing the course of mortality, likewise presents a disturbance at this time. The peak of mortality at 5 days also represents a critical period as indicated by the presence of a peak in the lactic acid curve.

These results are quite unexpected and no doubt, new. Students of animal growth have accepted the notion of Minot that the per-

centage rate of growth declines in a continuous manner with age. The breaks in the curves substantiate, in a way, the "human metamorphosis" conception recently advanced by Davenport.

Fig. 4 represents the course of carbon dioxide production plotted from data by other investigators. The differences between the curves in Figs. 2 and 4 are due to differences in experimental procedure.

Figs. 5, 6, and 7 represent the curves of growth in weight of the chick embryo. There are differences between the weight and the carbon dioxide curves, which leave room for discussion. There are also differences between the weight curves as plotted from data by different investigators. These differences are probably due to differences in the experimental technique employed, especially differences in incubation temperatures. That differences in temperature bring about changes in the growth rates, especially in the earlier stages of incubation, is illustrated by Fig. 8.

During postnatal life, the fowl grows at 5 per cent per day up to 3 weeks, and at 3 per cent from 3 to 12 weeks. The major inflection in the curve takes place at the age of about 12 weeks.

It may be noted in this connection that the rat, guinea pig, cow, sheep, and probably other domestic animals, grow at approximately the same percentage rate during the juvenile period (the stage preceding the major inflection); namely, 2 to 3 per cent per day. Man, however, grows during this period at quite a different (much lower) percentage rate.

The results obtained with the domestic fowl were practically duplicated, as far as the available data permitted, with the rat, guinea pig, cow, sheep, and domestic pig. The data of the rat are of special interest on account of the break in the curve at birth as shown in Fig. 9. It is probable that there is a break in the curve at birth in all classes of animals.

The curve of man differs in several important respects from the curve of animals. The curve of man requires a more extensive discussion than can be given at this time. For this reason a separate paper will be devoted to the growth curve of man.

The curve of plants is similar to that of animals. However, the inadequacy of the data and the relatively large experimental errors

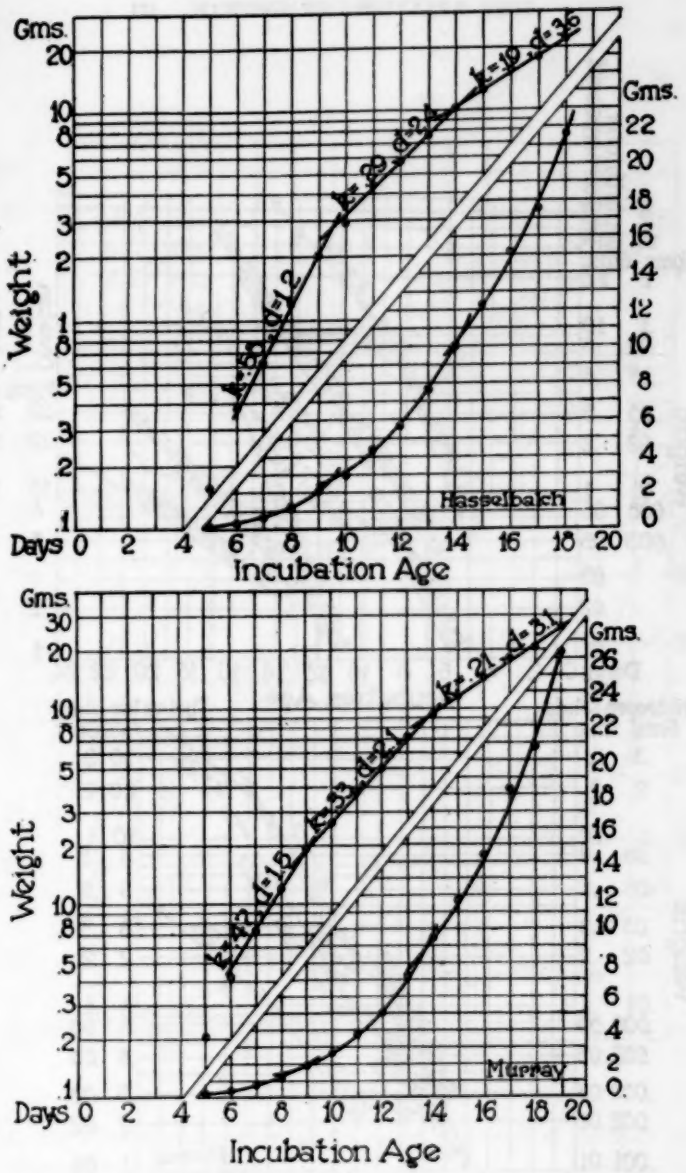

FIG. 6. Growth in wet weight of the chick embryo, plotted from data by Hasselbalch and by Murray. The value of 100 k represents the instantaneous percentage rate of growth per day. The values of d represent the time in days required for the body to double its weight.

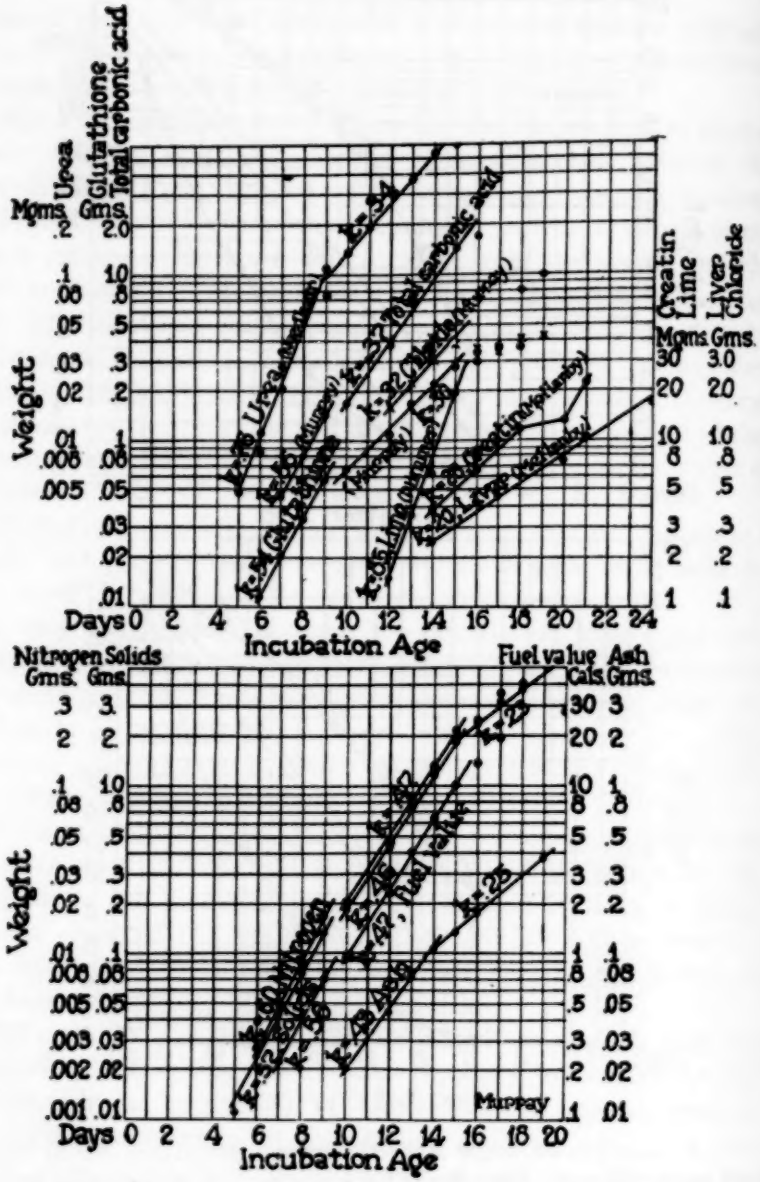

FIG. 7. The course of increase in chemical constituents in the chick embryo with advancing age. The sources of data are indicated on the chart (compare with Figs. 5 and 6).

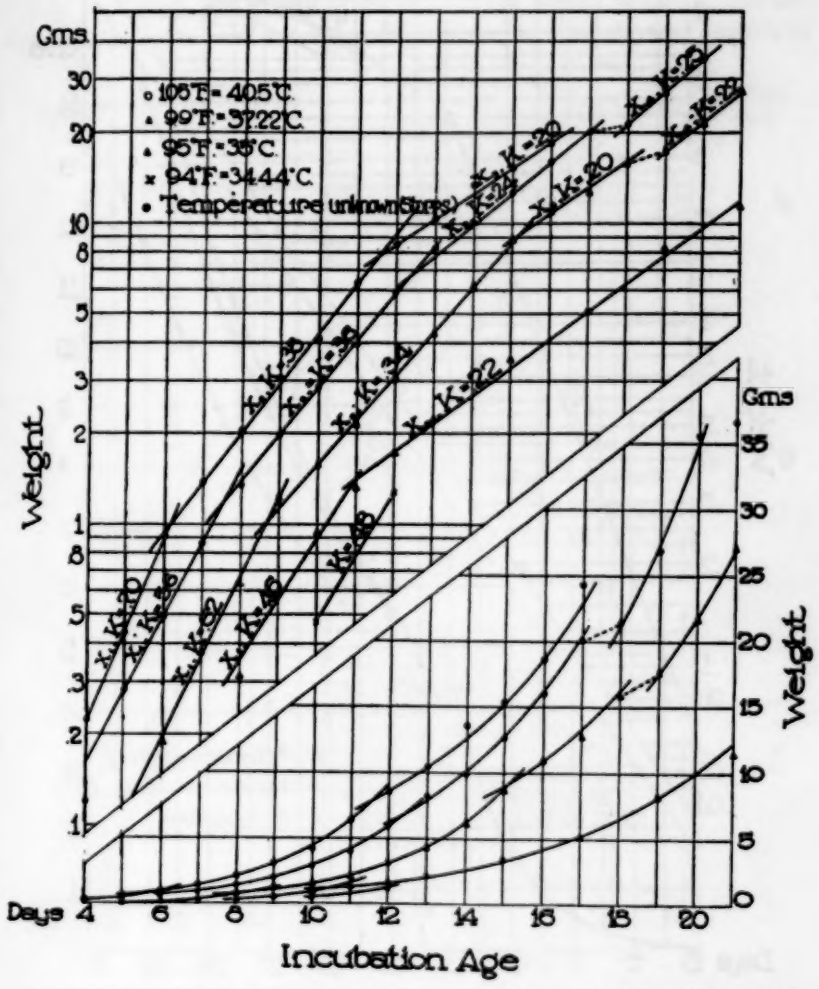

FIG. 8. The effect of temperature on the course of growth of the chick embryo (E. W. Henderson and S. Brody).

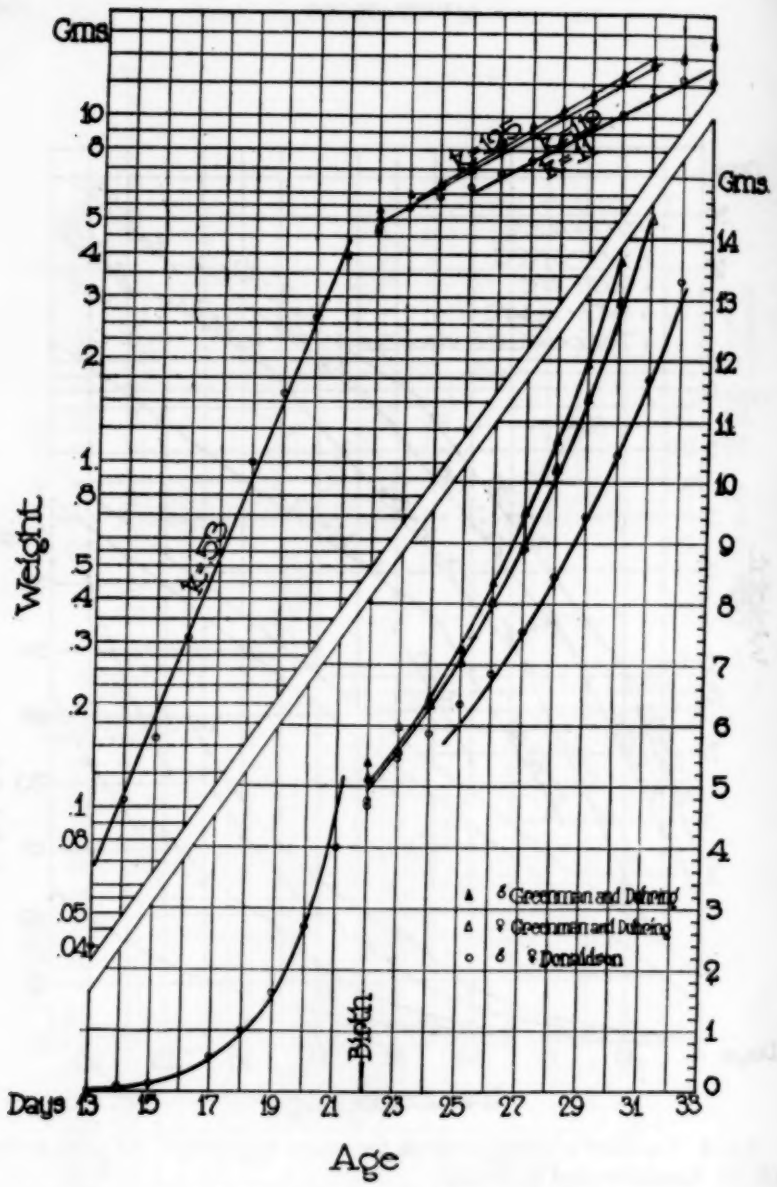

FIG. 9. The course of growth of the white rat plotted on arithlog and on coordinate paper. On the arithlog paper the data points are distributed around a straight line indicating that the percentage rate of growth is constant. There is an abrupt break in the curve at the time of birth and the percentage rate is seen to drop from 53 to about 12 per cent. Data preceding birth by Stotsenburg.

involved in the investigation of plant materials do not permit formulating conclusions as definite as with animals.

Fig. 10 shows the course of growth of the wheat kernel. The development of the seed corresponds, in time, to the prenatal growth in

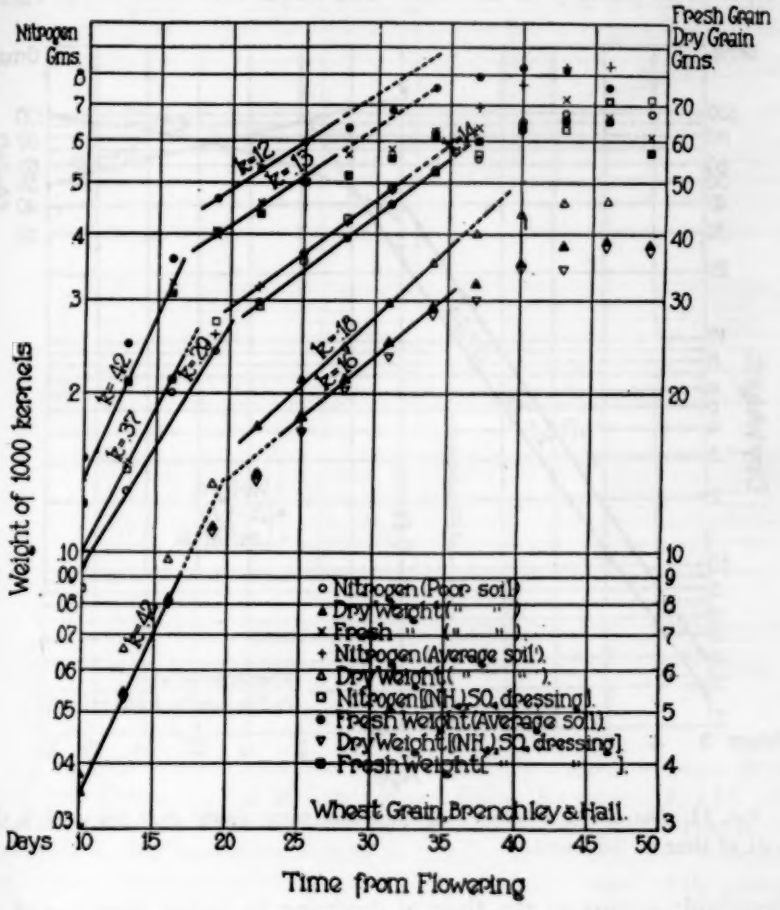

FIG. 10. Growth of the wheat grain. *k* and *d* have the usual meaning.

animals. However, it so happens that in the wheat kernel the embryo is a small fraction of the whole seed (about one-thirteenth), and so the data represent more than embryonic growth.

Fig. 11 represents the period of independent growth of the maize plant. The segment preceding flowering corresponds to the juvenile period in animals, and as in animals, the percentage rate of growth is constant. The inflection occurs at the time of flowering, which corresponds to puberty in animals. The major inflection in the curve

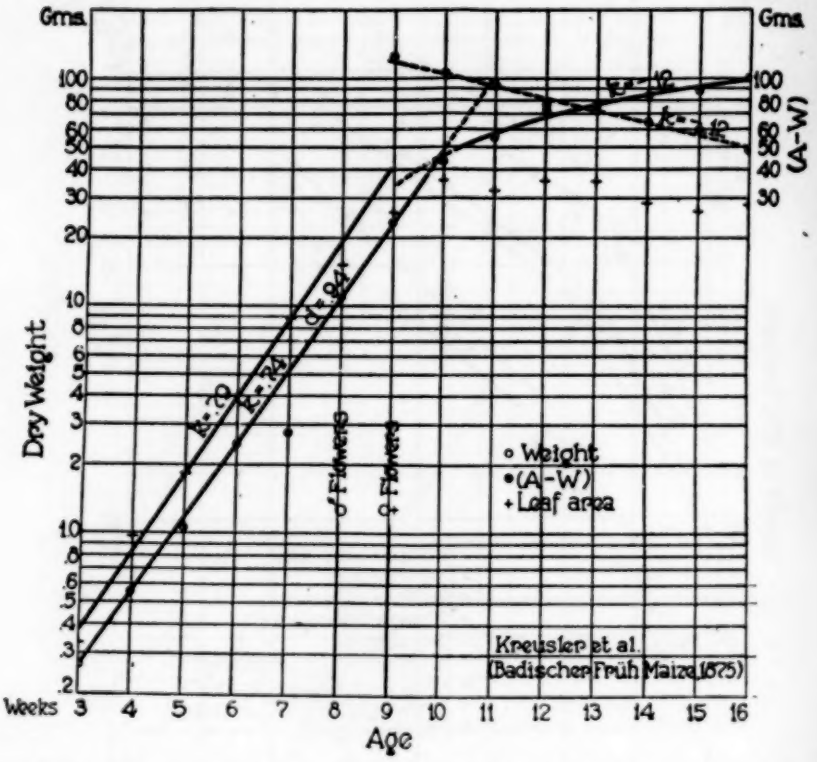

FIG. 11. The course of growth of the maize plant (note that the week is the unit of time in this case).

invariably occurs at the time of flowering in higher plants, and at puberty in higher animals.

Figs. 12 and 13 represent, respectively, the growth of bacterial and human populations. The rate of growth is constant during the period preceding the major inflection.

As to the bearing of this work on the problem of *growth cycles*, the

situation, as it appears to the writer, is as follows: All curves pass through an inflection which joins the strictly self-accelerating phase with the strictly self-inhibiting phase of growth. For this period,

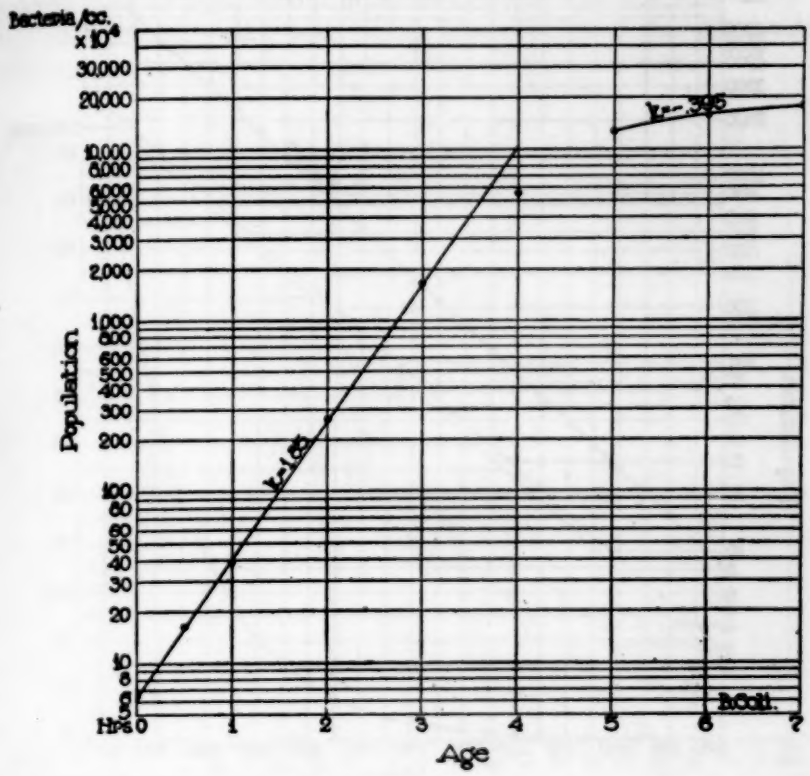

FIG. 12. The course of growth of *B. coli* at 37°C. in a given volume of broth (data from Experiment 7 of McKendrick and Pai). The value of k, 1.85, indicates the population of bacteria increases at 1.85 per cent per hour. That is, the population doubles itself every .69/1.85 = .37 hours, or 22.2 minutes. Following the 5th hour, the percentage rate of growth is constant with respect to the growth yet to be made.

which is relatively short, equation (6), the "autocatalytic" equation of Robertson, or the "logistic" equation of Pearl, can be fitted satisfactorily, especially, if a constant, or constants, is employed to compensate for the asymmetric nature of the curve. This equation can-

not, however, be satisfactorily fitted to the infantile (except in man), or to the juvenile cycle.

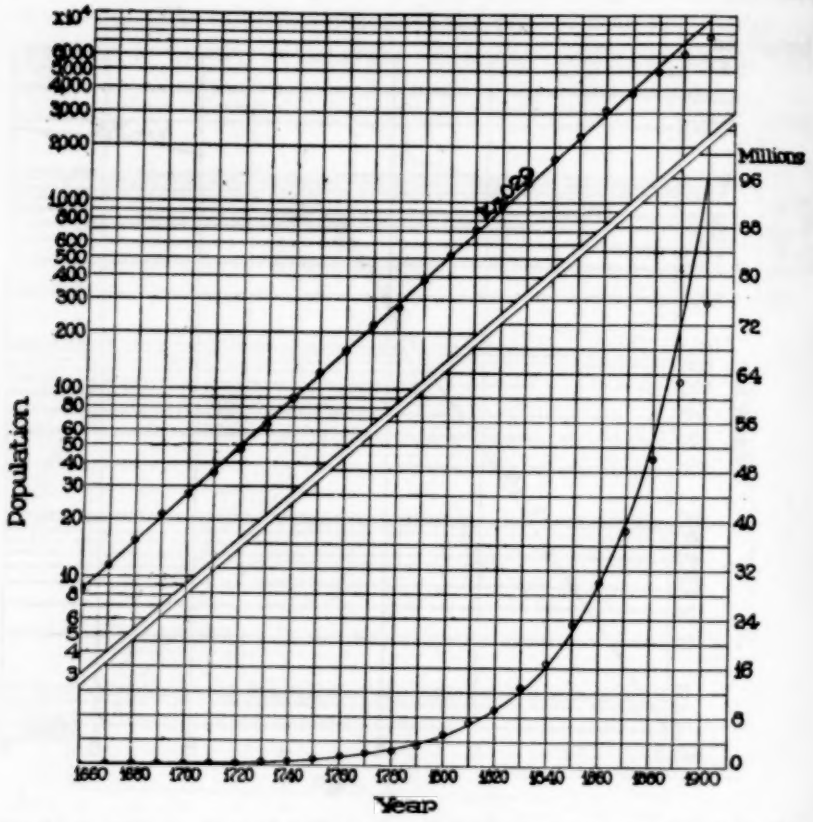

FIG. 13. The course of growth of the human population in the American Colonies and in the United States. The percentage rate is constant from 1660 to 1870. From 1870 on, the percentage rate declines in a manner indicated by the preceding figure on the growth of bacteria. $k = .029$; the population increased 2.9 per cent per year, or 29 per cent per decaded; or it doubled itself once in $.693/.029 = 24$ years. (Plotted from data by Rossiter, W. S., A century of population growth in the United States Bureau of the Census, United States Department of Commerce and Labor, Washington, 1909).

What we appear to have during the phase of growth preceding the inflection is a series of segments during each of which growth takes

place at a constant percentage rate. These segments are separated by breaks, analogous to the breaks in the curves of cold blooded animals when undergoing metamorphosis. The present need is for growth data taken at shorter intervals in order to ascertain definitely the presence of breaks, and for an investigation of the threshold mechanisms bringing about these breaks, if there are such.

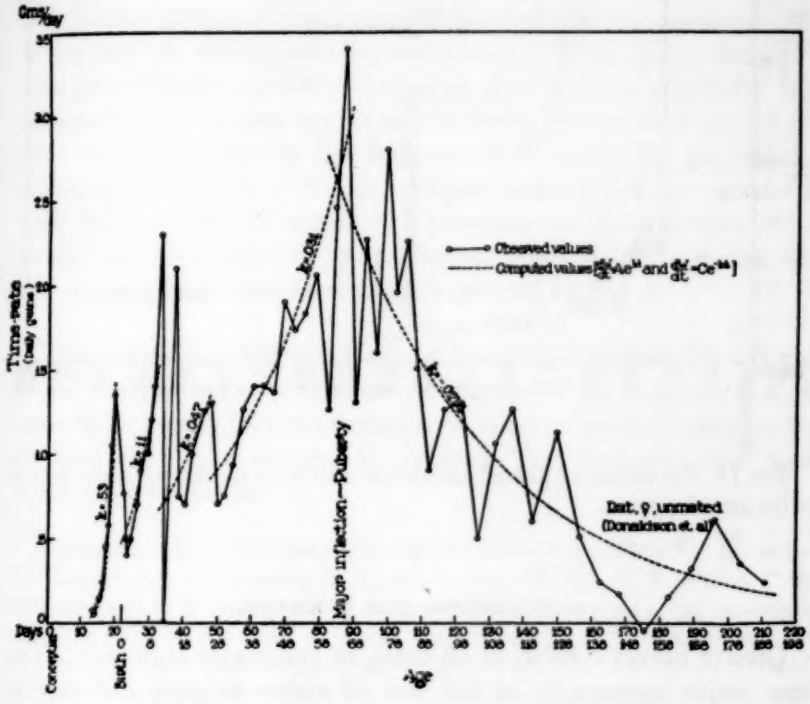

FIG. 14. The daily gains in weight of the rat plotted against age. The curve appears to have three cycles.

When the increments (time rates) are plotted against age, as shown in Fig. 14, there appear to be several cycles preceding the major inflection; as a matter of fact, the drops in the curve are not portions of cycles but breaks between successive stages of constant growth rates, as shown in Fig. 15.

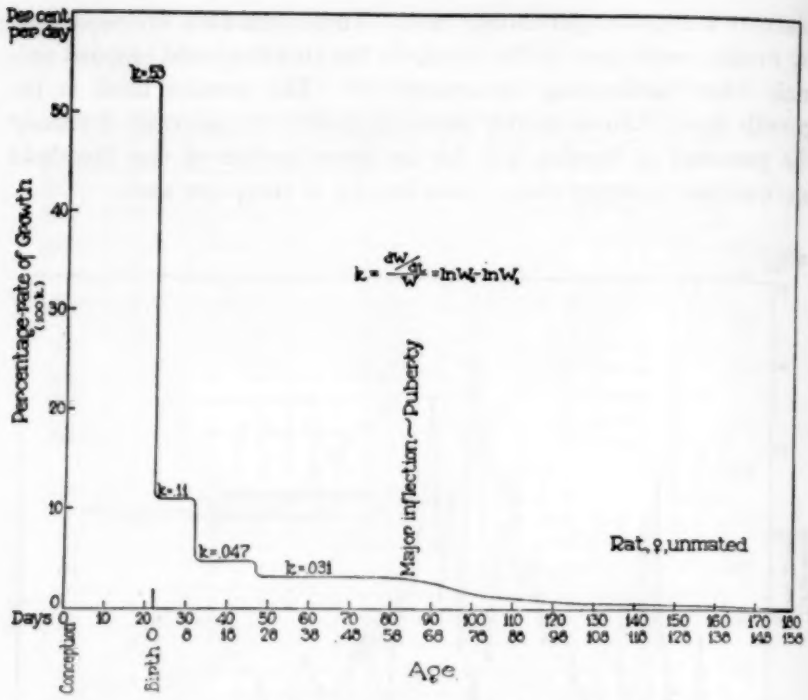

FIG. 15. The values of k plotted against age to indicate the discontinuous nature of the growth process.

V.

CONCLUSIONS AND SUMMARY.

Growth curves consist, in all cases, of two major segments. The first major segment is, in the case of higher animals and plants, made up in turn of several (probably five) shorter segments during each of which growth takes place at a constant percentage rate. The transitions between the successive stages are abrupt, the abruptness being of the order of metamorphosis in cold blooded animals.

It has been made clear in the first paper of this series that the time rate of growth following the major inflection *declines* at a constant percentage rate.

The junction between the two major segments occurs at puberty in animals and flowering in plants.

The two major segments are not symmetrical about the major inflection. The slope of the segment following the inflection is always less than the slope of the segment preceding the inflection. The major inflection does not occur in the center of the growth curve.

The instantaneous rate of growth at the beginning of growth is of the order of 100–200 per cent per day (*i.e.* the body weight is doubled in from 7 to 17 hours). It may be mentioned that 2 months after conception the rate of growth in man is only 8 per cent per day. This is contrary to all the published statements. Thus, Minot concluded that growth begins at 1000 per cent per day; Jackson concluded that in man, growth during the 1st month takes place at 57.5 million per cent per month; during the 2nd month 990 per cent per month; during the 3rd month 390 per cent per month (8 per cent per day is only 240 per cent per month). The reason for the discrepancy between the values derived, by the method adopted by the writer, and the values given in the literature is explained by Fig. 1.

This paper is a brief summary of Research Bulletins, 97, 98, and 99, of the University of Missouri Agricultural Experiment Station, at present in press. The reader must be referred to these bulletins for detailed discussions relating to questions that may not have been made clear in this paper.

Addendum.—Since this manuscript was submitted for publication, the writer had the privilege of discussing its subject matter with Drs. E. B. Wilson, C. R. Stockard, and H. H. Donaldson, all of whom expressed approval of the two principal ideas. Dr. Wilson called attention to a paper by G. H. Knibbs, on the Laws of population growth which appeared (on January 8) in the *Journal of the American Statistical Association*, 1926, xxi, 381, substantiating in principle one of the two principal ideas of this paper, namely that in the early history of a population the percentage rate of growth is constant. Dr. Stockard called attention to the fact that the peak in the mortality curve of the chick (Fig. 3) at 5 days is a counterpart of the peak in the prenatal mortality curve in man at 3 months. This is the junction between the embryonic period (formation of organs) and fetal period (enlargement of body and organs). The nature of the growth process in the two stages is quite different, and it is not, therefore, surprising to find a high mortality (and break in the growth curve) at this time.

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THE KINETICS OF EXOSMOSIS OF WATER FROM LIVING CELLS.

By MORTON McCUTCHEON and BALDWIN LUCKE.

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In previous communications data were presented on the rate with which water enters living cells (the unfertilized egg of *Arbacia punctulata*) under the driving force of osmotic pressure (1, 2). The present paper is concerned with the reverse process—exosmosis of water.

The material and technic of the experiments were the same as formerly employed.

The Kinetics of Exosmosis.

The first point was to determine whether exosmosis follows the same diffusion equation as does endosmosis, namely $\frac{dx}{dt} = k(a - x)$, where a is the total volume of water that will cross the membrane before equilibrium is established, x the amount that has already crossed at time t, and k is the velocity constant. For this purpose, eggs were placed in a dish containing 60 per cent sea water (sea water 60 parts, and distilled water, 40 parts). In this hypotonic solution, eggs were allowed to swell until osmotic equilibrium was attained. A number of eggs were then transferred to a second dish containing full strength sea water (100 per cent sea water). Three cells were measured with an ocular screw micrometer at minute intervals, until they had again reached osmotic equilibrium. Duplicate observations were usually made by the two observers. The mean volumes of 6 or more cells were plotted against times, and a curve obtained, as is shown in Fig. 1. In the same graph, $\log \frac{a}{a - x}$ is plotted against times. This plot is found to give a straight line, the slope of which is k, the velocity constant.

It is evident from this relation that the process of exosmosis follows the same equation as was previously found to fit endosmosis. This result was invariably obtained in scores of experiments at various temperatures.

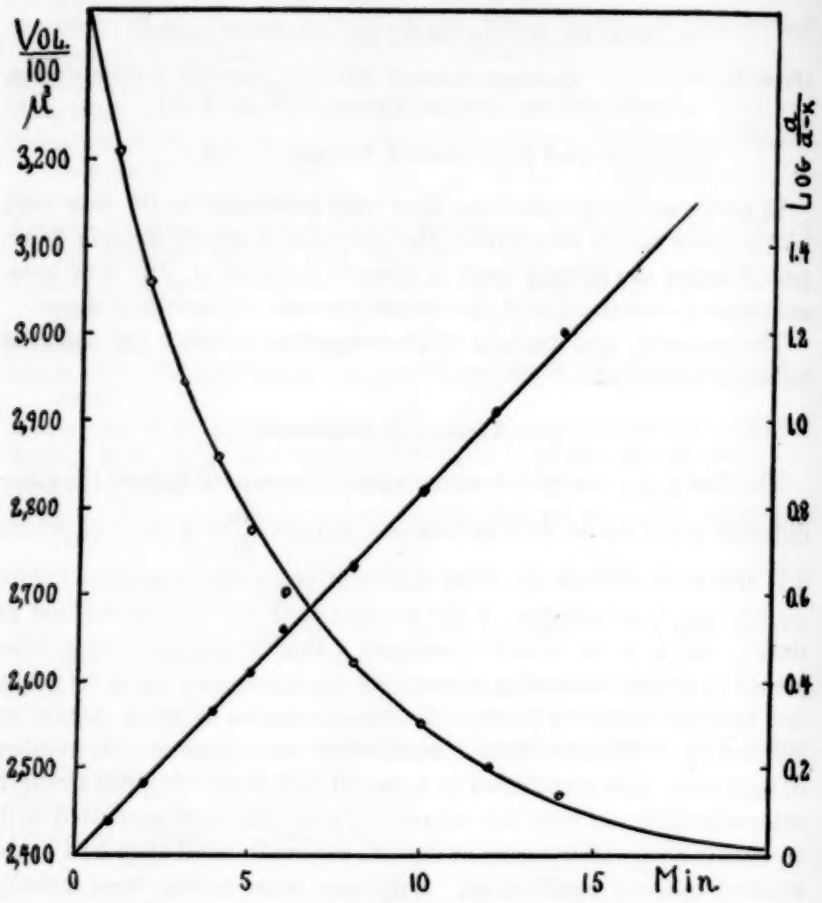

FIG. 1. A typical exosmosis experiment. Cells previously swollen in 60 per cent sea water were caused to shrink by placing them in 100 per cent sea water at 15°C. The curve represents the decrease of volume with time (open circles). The graph of $\log \frac{a}{a-x}$ against time (solid circles) is a straight line, the slope of which gives the value of k (= 0.085). This graph shows that the process follows the equation $kt = ln \frac{a}{a-x}$. (Each point represents the mean of 10 cells.)

Relative Rates of Exosmosis and Endosmosis.

The second point was to determine whether the *rates* of exosmosis and of endosmosis are the same. For this purpose eggs were placed in 60 per cent sea water, and velocity constants of swelling determined at several temperatures, as previously described (1). Eggs from the same animal were then returned from 60 per cent sea water to 100 per cent and the constants for the reverse process obtained. The results of

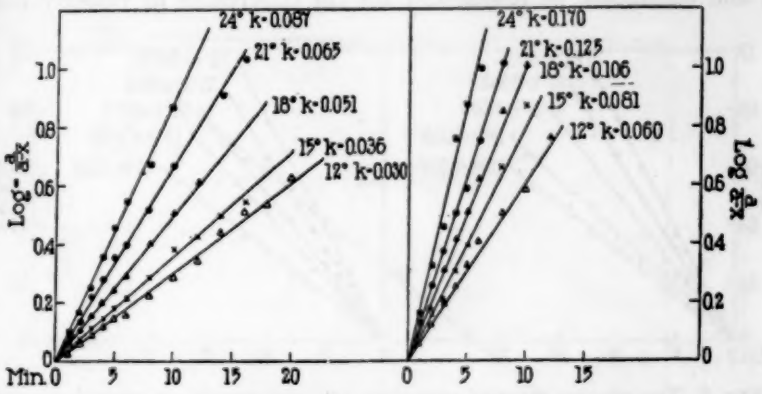

FIG. 2. The relative rates of exosmosis and endosmosis, at several temperatures. Log $\frac{a}{a-x}$ is plotted against time. The resulting graph is for each temperature a straight line, the slope of which gives the value of k, the velocity constant.

The graphs on the left represent *endosmosis* (swelling in 60 per cent sea water); the graphs on the right, *exosmosis* (shrinking after return to 100 per cent sea water). Under these conditions the values of k are, at each temperature, approximately twice as great in the latter process (contrast with Fig. 3). (Each point represents the mean of six cells.)

a typical experiment are shown in Fig. 2, in which $\log \frac{a}{a-x}$ is plotted against times, for both endosmosis and exosmosis. It is seen that at a given temperature, the velocity constant of exosmosis is greater than that of endosmosis.

This experiment was repeated a number of times. While the values of k varied with different lots of eggs, essentially similar results were

practically always obtained: the eggs shrank faster than they had swollen.

Subsequent experiments, however, showed that this difference in rate between exosmosis and endosmosis was not an essential one, that it existed only under special conditions. In the experiments just reported 60 per cent sea water was used to produce swelling of cells, but another concentration—100 per cent—to produce shrinking. Might this fact, rather than essential dissimilarities between endosmosis and exosmosis, be responsible for the differences in velocity con-

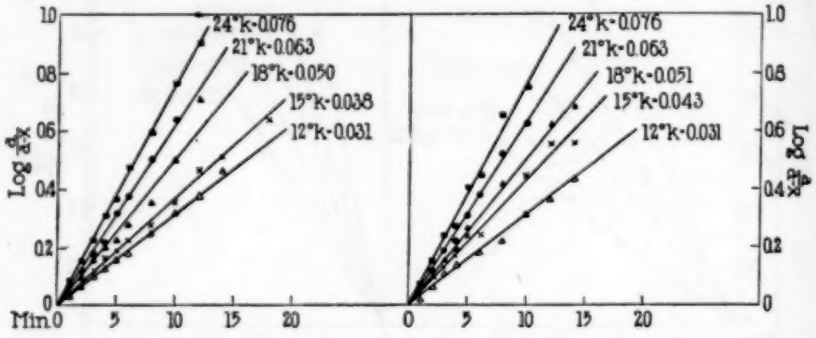

FIG. 3. The relative rates of exosmosis and endosmosis, at several temperatures, in media of the *same* osmotic pressure. The graph on the left represents *endosmosis* (swelling in 60 per cent sea water); the graph on the right *exosmosis* (shrinking in 60 per cent sea water after previous swelling in 50 per cent sea water). The medium is therefore of the same concentration in both processes; under these conditions the values of k are almost identical in exosmosis and endosmosis at each temperature (contrast with Fig. 2). (Each point represents the mean of six cells.)

stants? If swelling and shrinking could be produced in solutions of the *same* concentration, it seemed possible that equal values of k would be obtained.

Accordingly, eggs were placed in 60 per cent sea water at various temperatures and the several velocity constants determined for swelling. Other eggs were placed in a *more* hypotonic solution—50 per cent sea water. After having attained constant volume they were transferred to a 60 per cent solution and allowed to shrink, values of k being obtained as before. The results of this experiment are shown in Fig. 3. It is seen that the slope of the lines is the same for shrink-

ing as for swelling, indicating that the velocity constants for the two processes, at each temperature, are the same.

Similar results were obtained where cells were allowed to swell in 70 or 80 per cent sea water, and other cells were allowed to shrink in solutions of the same concentration. In every experiment, practically identical values of k were obtained for swelling and shrinking at a given temperature, providing that the concentration of the medium was the same.

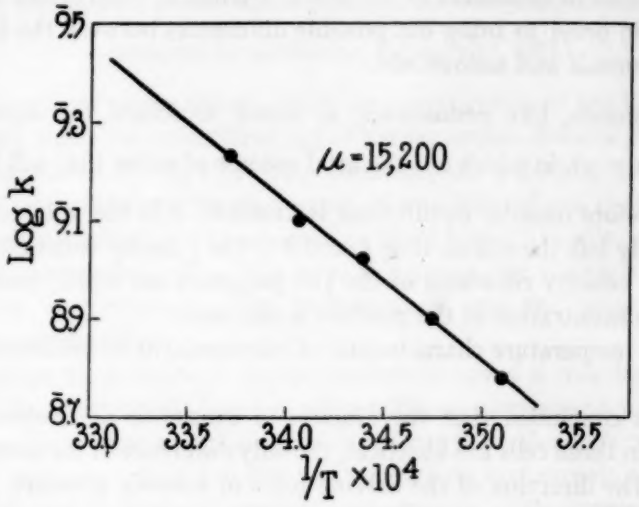

FIG. 4. The effect of temperature on exosmosis. The logarithms of the velocity constants of the experiment shown in Fig. 2 (graphs on the right) are plotted against the reciprocals of the absolute temperature. The slope of the line gives the value of μ (= 15,200).

Effect of Temperature on Exosmosis.

It follows from this as a corollary that exosmosis is affected by temperature just as is endosmosis (1), that is, there is a marked increase in rate with rise in temperature. Fig. 4 shows that the Arrhenius equation holds, the value of the temperature characteristic in this case being 15,200 (data were taken from Fig. 3). The value of μ varied in different experiments much as in endosmosis, but was always high.

These experiments therefore appear to indicate that the kinetics of exosmosis and endosmosis of water in this material are identical. The only difference in the processes is that the direction of the driving force of osmotic pressure is reversed. Both processes are affected in the same way by the external factors of temperature and concentration of sea water.

SUMMARY.

1. The rate of exosmosis of water was studied in unfertilized *Arbacia* eggs, in order to bring out possible differences between the kinetics of exosmosis and endosmosis.

2. Exosmosis, like endosmosis, is found to follow the equation $\frac{dx}{dt} = k\,(a - x)$, in which a is the total volume of water that will leave the cell before osmotic equilibrium is attained, x is the volume that has already left the cell at time t, and k is the velocity constant.

3. The velocity constants of the two processes are equal, provided the salt concentration of the medium is the same.

4. The temperature characteristic of exosmosis, as of endomosis, is high.

5. It is concluded that the kinetics of exosmosis and endosmosis of water in these cells are identical, the only difference in the processes being in the direction of the driving force of osmotic pressure.

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THE EFFECT OF SALT CONCENTRATION OF THE MEDIUM ON THE RATE OF OSMOSIS OF WATER THROUGH THE MEMBRANE OF LIVING CELLS.

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The present paper reports a further investigation of the kinetics of osmosis, using the unfertilized egg of the sea urchin *Arbacia punctulata* as an osmometer. In a previous communication dealing chiefly with the affect of temperature on osmosis, it was pointed out that the rate of osmosis is influenced by a second external factor, the salt concentration of the medium (1). It is with this factor that the present investigation is concerned. The technic used was the same as that previously described.

In order to understand the mechanism involved it was desirable to study the effect of salt concentration of the medium over a wide range —from 20 per cent to 125 per cent of sea water.[1] Hence it was obviously necessary that for low concentrations endosmosis should be studied, and for high concentrations exosmosis. This procedure was justified since both processes follow the same diffusion equation,[2] and since their velocity constants are practically identical when the concentration of the medium is the same in the two cases (2).

Experiments.

One preliminary question needed to be answered. Suppose different lots of eggs (from the same animal) were placed in 60, 70, 80 and 100 per cent sea water

[1] Sea water evaporated so that the concentration of salts is 5/4 that in 100 per cent sea water; the original pH of this solution was restored with CO_2.

[2] $\frac{dx}{dt} = k\,(a - x)$, where a is the total volume of water that will cross the membrane before equilibrium is established, x the amount that has already crossed at time t, and k is the velocity constant.

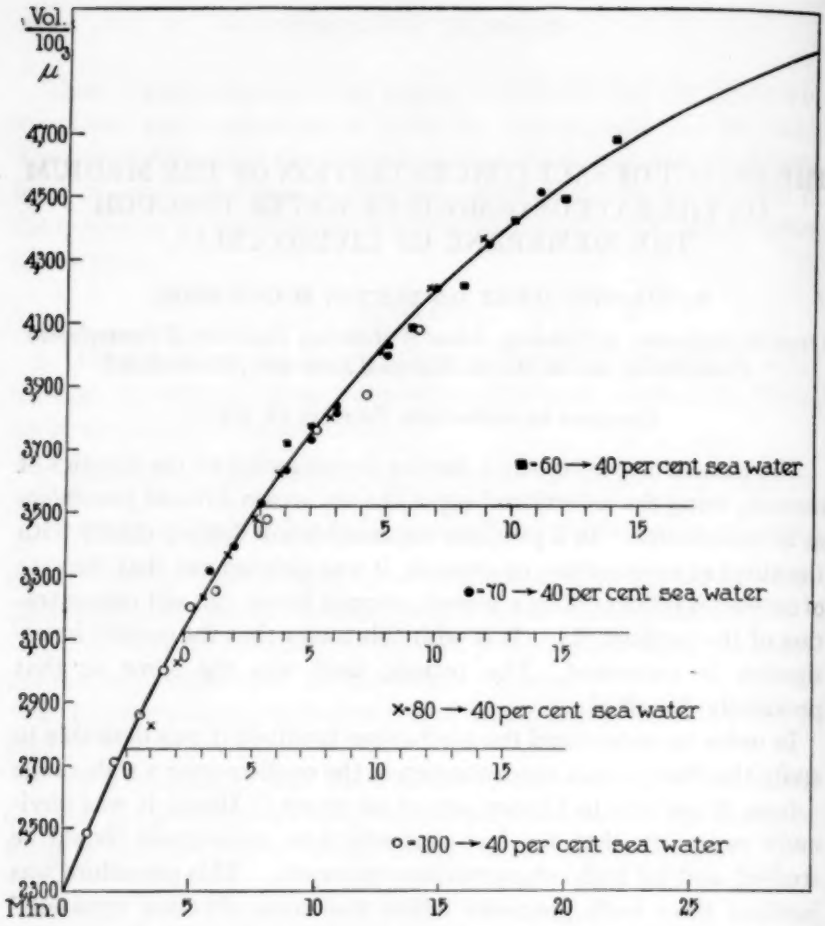

FIG. 1. Different lots of cells were placed in 60, 70 and 80 per cent sea water and allowed to reach osmotic equilibrium; then each lot was transferred to 40 per cent sea water, in which further swelling occurred. In each case the observational points from the several experiments fall along a single curve calculated from the equation $kt = \log \frac{a}{a - x}$. (In this equation the value of a was calculated as previously described (1); the value of $k = 0.027$ was determined by transferring cells from full strength—100 per cent—sea water to 40 per cent sea water.)

The points of intersection of the several base lines with the curve were found by observing the mean volume at equilibrium of twenty cells in 80, 70 and 60 per cent sea water, respectively; these values correspond to volumes at time zero in the several experiments.

The values of k in the several experiments were practically the same as the value of k for the curve. (Each observational point represents the mean of six cells.)

and allowed to reach osmotic equilibrium, and then each lot was transferred to a more hypotonic solution, *e.g.* 40 per cent sea water, in which further swelling would occur, would the values of the velocity constants be the same in all lots,—*i.e.* would volume increase go half way to equilibrium in the same length of time? Several such experiments were made, and an affirmative answer was obtained. Thus the values of k for swelling, on transferring the cells from the several solutions into 40 per cent sea water at 21°C., were as follows: from 100 per cent, 0.027; from 80

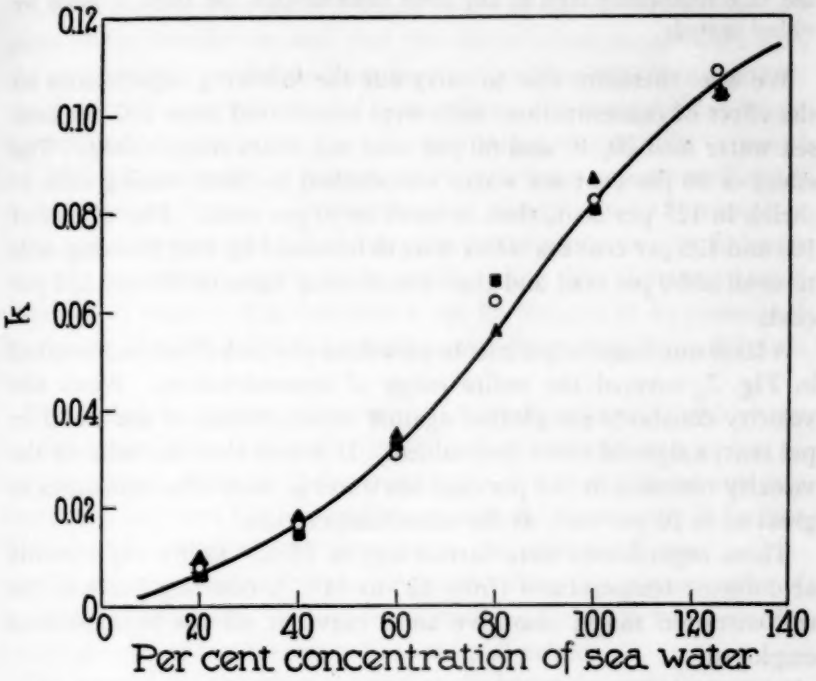

FIG. 2. The effect of osmotic pressure of the medium on the rate of osmosis of water through the membrane of living unfertilized *Arbacia* eggs. The values of k, the velocity constant, of three separate experiments represented by different symbols are plotted against the concentration, in per cent, of sea water. A sigmoid curve is thus obtained. (Each point represents the mean of six cells.)

per cent, 0.028; from 70 per cent, 0.028; from 60 per cent, 0.026. The differences are not regarded as significant. The results are plotted in Fig. 1. In each case the observed points from the several experiments fall along a *single* curve calculated from the equation $kt = \log \frac{a}{a - x}$, as described in the legend. It is seen that the curves for cells taken from 60, 70 and 80 per cent sea water are merely upper segments of the curve for cells taken from 100 per cent sea water.

Similar results were obtained on causing cells to *shrink* on being transferred from various solutions into 100 per cent sea water at 19°C., *i.e.* the values of the velocity constants were practically the same, though more variation was encountered. The results were as follows: from 40 per cent, $k = 0.15$;[3] from 50 per cent, 0.13; from 60 per cent, 0.14; from 70 per cent, 0.12; from 80 per cent, 0.15.

This point established, the problem was greatly simplified. We were justified in causing cells to swell or shrink in any convenient concentration of sea water, and then transferring them to any other concentration, the effect of which we wished to study.

We were therefore able to carry out the following experiments on the effect of concentration: cells were transferred from 100 per cent sea water into 20, 40 and 60 per cent sea water respectively. The effect of 80 per cent sea water was studied by first causing cells to shrink in 125 per cent, then to swell in 80 per cent. The effects of 100 and 125 per cent sea water were determined by first allowing cells to swell in 50 per cent and then transferring them to 100 and 125 per cent.

A large number of experiments were done of which three, represented in Fig. 2, covered the entire range of concentrations. When the velocity constants are plotted against concentration of sea water in per cent, a sigmoid curve is obtained. It is seen that the value of the velocity constant in 125 per cent sea water is more than ten times as great as in 20 per cent, at the same temperature.

These experiments were carried out at 15°C. Other experiments at different temperatures (from 12° to 24°C.), covering parts of the concentration range, also give an S curve at all the temperatures employed.

DISCUSSION.

The experiments show that the velocity constant of the diffusion process is altered by change in the concentration of the medium. As the medium is made more dilute (by the addition of distilled water), the velocity constant becomes smaller, and, conversely, increase in the concentration of the medium leads to increase in the velocity constant (Fig. 2). At any concentration, the rate of the process still obeys the fundamental diffusion equation, $kt = ln \frac{a}{a - x}$. But the

[3] The eggs had not reached equilibrium in 40 per cent sea water.

fact that the value of k depends on the concentration of the medium, points to another factor modifying the rate of diffusion. This factor would seem to be a change in permeability of the membrane to water. It appears that dilution of the medium decreases permeability to water, while concentration increases it.

The mechanism involved may be pictured as follows:

Let us assume that water diffuses into and out of the cell through pores in the membrane, and that the size of these pores varies with the concentration of the medium, in the sense that they become larger the greater the concentration of the medium. Such a condition might be explained by regarding the cell membrane as a partially hydrated gel, which takes up water from *hypotonic* solutions, and gives off water in *hypertonic* solutions. In the one case, the gel can be imagined to swell and so reduce the size of its pores, in the other case to shrink so that the pores become more widely opened, and thus allow more rapid diffusion of water. The membrane can be thought of as permeated by the outside solution and instantly coming into osmotic equilibrium with it.

The S shape of the concentration effect can now be explained by the further assumption that the hydrophilic membrane takes up water at first to a great extent as the concentration of the outside sea water is decreased, but then to a less and less extent as the sea water is further diluted and the membrane approaches the saturation point. Conversely, the same reasoning can be applied when the concentration of sea water is increased—at first the membrane yields its water readily but at greater concentration only to a small extent.

This hypothesis therefore states that the lower the osmotic pressure of the sea water, the less permeable is the membrane to substances passing through its pores, and conversely. Such a theory is apparently capable of being tested experimentally.

SUMMARY.

1. Using the unfertilized egg of the sea urchin, *Arbacia*, as osmometer, it was found that the rate with which water enters or leaves the cell depends on the osmotic pressure of the medium: the velocity constant of the diffusion process is higher when the cell is in concentrated sea water, and lower when the sea water medium is diluted with dis-

tilled water. Differences of more than tenfold in the value of the velocity constant were obtained in this way. When velocity constants are plotted against concentration of medium, a sigmoid curve is obtained.

2. These results are believed to indicate that cells are more permeable to water when the osmotic pressure of the medium is high than when it is low. This relation would be accounted for if water should diffuse through pores in a partially hydrated gel, constituting the cell membrane. In a medium of high osmotic pressure, the gel is conceived to give up water, to shrink, and therefore to allow widening of its pores with more ready diffusion of water through them. Conversely, in solutions of lower osmotic pressure, the gel would take up water and its pores become narrow.

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STUDIES ON THE PERMEABILITY OF MEMBRANES.

II. Determination of Ionic Transfer Numbers in Membranes from Concentration Chains.

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1. THEORY.

It has been shown (1) that the P.D. of a concentration chain, *e.g.* with two KCl solutions in the ratio 10:1, across a membrane of dried collodion, reaches its maximum value better the more dilute the KCl solutions are, and that in higher ranges of concentrations, the effect of the membrane vanishes more and more. So in the latter case the P.D. approaches closely that established without a membrane. This is, in the case of KCl, practically equal to zero.

The interpretation of the effect of the membrane was given as follows: Any potential difference between two aqueous solutions of different electrolytic content, in direct contact with each other, is due to the difference in the mobilities of the different kinds of ions. In the ultracapillary spaces of the membrane, the mobilities of the ions are not only changed with respect to their absolute value, but also to their relative value. In general, the mobility of the anion is considerably more decreased than the cation.

Let us consider a very thin layer of the liquid content within a pore channel confined by two cross-sections through the channel, infinitely close to each other. Let the two sides of the membrane be in contact with two different solutions of the same kind of electrolyte consisting of two univalent ions. A transition zone will be formed across the membrane. Then the P.D. between two infinitely close neighboring cross-sections through this transition zone will be:

$$dE = \frac{RT}{F}\frac{u - v}{u + v}\frac{dc}{c} \qquad (1)$$

where u and v are the mobilities of the cation and anion, respectively, within the concerned cross-section; c is the concentration, or better, the activity of the electrolytes within this infinitely thin layer of the liquid, and dc the change of c from the one cross-section to the neighboring one. In order to find the total P.D. from the one side of the membrane to the other, the above formula must be integrated over all the infinitely small cross-section layers from the one side of the membrane to the other. Let us designate the direction along the axis of the pore channel as the x axis; the integration has to be performed from $x = 0$ to $x = D$, where D is the total thickness of the membrane. Now, from the experiments referred to, we have to infer that u and v within the channel depend considerably on the concentration in a degree much more pronounced than in a free aqueous solution. Usually in an aqueous solution u and v depend only slightly on the concentration. Here, with a certain approximation, we may consider u and v as constant, and the result of the integration becomes:

$$E = \frac{RT}{F} \cdot \frac{u - v}{u + v} \ln \frac{C_2}{C_1} \tag{2}$$

when C_2 and C_1 are the two concentrations of the solution which are in contact with each other and connected by a transition zone. It should be emphasized that this result of the integration does not depend on the manner in which the transition of the one concentration to the other is realized. It does not depend on whether the fall of the concentration is rectilinear or arranged in some other way; whether the transition is realized by a pure diffusion zone or by a zone of mechanical mixtures of the two solutions.

However, within the membrane there is no constancy of u and v over the range of integration, u and v depend strongly on c, and, therefore, also on x. In the integration, the factor containing u and v must not be placed before the integration sign, and the result is:

$$E = \frac{RT}{F} \int_{x=0}^{x=D} \frac{u - v}{u + v} dc \tag{3}$$

If, instead of this accurate formula, we apply formula (2), the letters u and v no longer have the meaning of the mobility of the cation and anion, respectively, but they may be interpreted as some kind of

average mobility,[1] within the range of concentration C_1 and C_2. Such an average mobility has no simple physical meaning. It need not be the arithmetical mean.

In the ordinary concentration chain without a membrane, we are allowed to calculate the relative value of u and v, or the transfer numbers $t^+ = \frac{u}{u+v}$ and $t^- = \frac{v}{u+v}$ from the measurement of the P.D. availing ourselves of the formula (2) which solved for t^- reads, for a temperature of 20°C.:

$$t^- = 0.5 - \frac{E}{2 \times 58 \times \log^{10} \frac{C_2}{C_1}} \qquad (4)$$

where E is the measured P.D. in millivolts.

If we apply this formula to a concentration chain with a membrane, the obtained transfer number of the anion, t^-, is nothing but the calculated average value of t^-, which has not a simple physical meaning. However, when the ratio $C_2:C_1$ is small enough, the variation of t^- for the concentration C_2 and C_1 may be also small, so that with a certain approximation the calculated t^- may be regarded as a useful approximation to both the value of t^- for C_2 and for C_1. Using this idea, there is obtained a method for calculating, with a certain approximation, the transfer number of the ions of an electrolyte within the pores of the membrane for any concentration of the aqueous solution in contact with the membrane and in equilibrium with the electrolytic content of the membrane pores.

The method consists in the measurement of the P.D. of concentration chains with a membrane, the ratio of concentrations of the two solutions being as small as possible. It is not practical to apply very small ratios, for in this case the measured P.D. becomes very small also and

[1] It should be borne in mind that according to the interpretation given in the previous paper, even the mobility of an ion, especially of an anion, within *one* very thin cross-section layer of a channel, is but an average value, as the anions fixed at the wall and the free anions in the center of the pore have different mobilities. The average value of these mobilities is called here the mobility of that ion within this cross-section. In this present paper the "average" mobility of an ion means the average of the thus defined mobilities, over the range of all of the cross-sections through the channel from its one opening to the other.

the inevitable error of, let us say 0.5 millivolt, which may occur in the measurement of such a chain and which may be due to uncertain and irreproducible liquid junction potentials, has too high an influence on the results. The best ratio of concentrations seemed to be the one of 2:1. The maximum effect, when the mobility of the anion = 0 is in this case 17.6 millivolts for 20°C.[2]

Several previous authors emphasized the fact that the transfer numbers are altered in membranes. As all of these authors applied methods different from those used in this paper, and as review of these findings is to be made in a subsequent paper, we refrain here from discussing the literature, in order to avoid repetition, especially because the essential point,—the rapid change of the transfer number according to the concentration, has not yet been described so far as we know.

2. Technique.

The experiments were carried out with the dried collodion bag membrane, as described in the previous paper. Some series were made with flat membranes which have advantages to a certain extent; particularly in that they maintain their original properties as measured by the *Co P* virtually permanently. Such flat membranes were used by the authors through many successive experiments over months, the *Co P* having remained constant within some tenths of a millivolt. Flat collodion membranes have been used in the past, *e.g.* by Bethe and Toropoff (2), Bigelow and Gemberling (3), Bartell and Carpenter (4), and Hitchcock (5), and they have been used also long ago for ultrafilters by Bechhold, Zsigmondy, and others. But these authors worked with more or less well permeable, not completely dried collodion membranes. It is very easy to obtain this kind of membrane in a flat form, but it requires special technique to make completely dried membranes in a flat form. Recently Collander (6) described the flat form for the dried collodion membrane emphasizing its great stability. The difficulty consists in the fact that the flat membranes, while drying, shrink and shrivel and become inelastic and rigid, so

[2] The error involved in the fact that a concentration ratio is used instead of one between activities, is so small for a ratio of 1:2 as to be negligible with respect to other errors involved.

that they cannot be fastened without a leak to any opening of a vessel. One has to fix the membrane, in a half dried condition, to the rim of a vessel and then allow it to dry. The authors found the following method best.

A 5 per cent solution of celloidin Schering in 75 per cent ether and 25 per cent alcohol was poured on a mercury surface in a Petri dish, as Bartell suggested, in an amount sufficient to cover easily the whole surface by the spontaneous spreading of the viscous solution. The collodion is allowed to dry to such an extent that the consistency is just suitable for cutting with a knife. At this time, the periphery adjacent to the rim of the Petri dish is cut and the membrane taken out and put over the opening of a bell jar shaped glass, such as is shown in Fig. 1. The diameter is 2 cm. smaller than that of the Petri dish in a cross-section. The rim is

FIG. 1. Glass bell jar used as supporting frame for flat membranes.

represented by an even ring 1 cm. broad with ground surface. The rim is wet with a layer of collodion solution immediately before the half dried membrane is put on. By means of this the membrane is glued on the rim. While the drying process is going on, the main part of the membrane covering the opening of the jar is prevented from shrinking. However, the peripheral portion of the membrane shrinks in such a way that a circular sulcus outside the glass rim is formed, the concavity being directed toward the pointed part of the jar. Now the jar is turned over and collodion solution is poured into this sulcus. This fills in the leaks which may be left between the membrane and the glass rim. Moreover, while drying and shrinking, the peripheral collodion ring shrinks and rolls so that finally a tight band hermetically seals all leaks. The membrane has to stand 3 or 4 more days to complete dryness which can be recognized by electrification after rubbing with hair. The membrane is exposed to an enormous

stress, and too thin a membrane sometimes will crack. Those membranes which have withstood the stress have turned out to be virtually invariable in any respect. The *Co P*, even after months of experiments, remains constant within a fraction of a millivolt. It is true, in consequence of the complete lack of shrinking, the *Co P* does not reach such high values as sometimes is obtained in the shrunk bag membranes. Usually a *Co P* of about 48 millivolts was obtained, whereas the other form sometimes gives 52 to 53 millivolts. In the second place, on account of the thickness, a long time is necessary to reach a stationary condition when measuring the P.D. To measure, for example, the *Co P*, the membrane has to stand at least some hours in contact with the solution, the latter being several

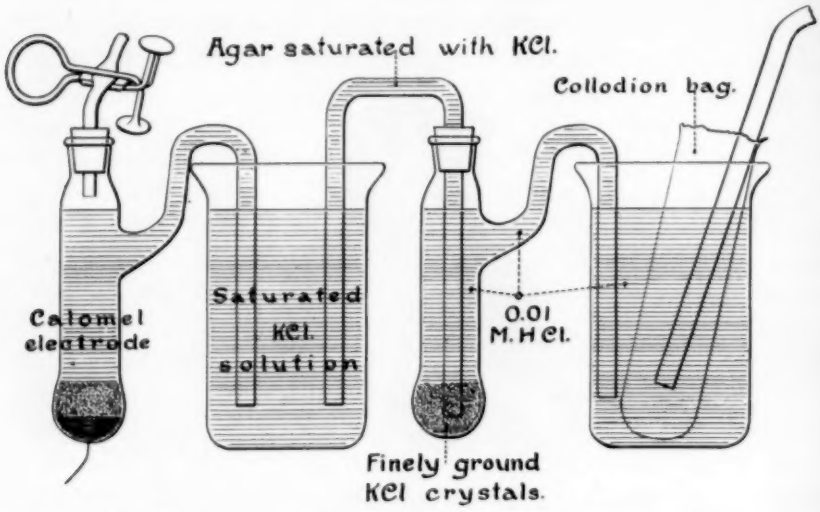

FIG. 2. Method of connecting calomel half-cell with membrane system to avoid diffusion potentials and diffusion.

times renewed. However, after the stationary condition has been established, the P. D. keeps astonishingly constant for any period.

When a membrane used for one experiment is to be used for another with different solutions, the previous solutions must be washed out at least 24 hours; and in certain cases, *e.g.* in the transition of KCl chain to a LiCl, where any trace of the much more movable K will spoil the value of the Li chain, the washing process should be prolonged several days. Inconvenient as that may be, the labor is rewarded by very constant and reproducible results, and on account of its invariability,

such a membrane can be used for comparative successive experiments with different solutions much better than the bag membrane.

As an error of even 1 millivolt has a relatively great influence on the calculation of the transfer numbers, the errors involved in liquid junction potential should be eliminated as far as possible. In chains with KCl there is no trouble in this respect, but for other electrolytes the arrangement shown in Fig. 2 was used. This represents a .01 M HCl solution outside the membrane connected with the leading off calomel half cell. The same arrangement was used to connect the inside solution of the collodion bag with the other electrode. This arrangement is based on the principle of Michaelis and Fujita (7), interposing between the HCl solution and the saturated KCl solution another HCl solution of the same concentration as the first one, but also saturated with KCl. We may be sure that the liquid junction potential is reduced to a fraction of 1 millivolt at least. The two calomel half cells, saturated with KCl, were often checked against each other and when a small P.D. was observed between them, a suitable correction for the P.D. of the chains in the experiments was made.

3. Results and Discussion.

Table I shows the P.D. of a chain with a dried collodion bag membrane between two KCl solutions of the concentration 1:2, in different ranges of concentration. The last column gives the transfer number for Cl evaluated according to formula (4) (Fig. 3, lower curve).

Table II shows a similar experiment with a flat membrane. Here the constancy of all values is best, though long time and repeated change of the solution was necessary to reach these constant potentials. The definite value of this series is most trustworthy of all, and the smoothness of the interpolation curve (Fig. 4) confirms this assumption.

Table III gives a series of experiments with a fairly stable bag membrane and with the chlorides of H, K, Na, and Li. The entire series would have consumed too much time, had it been made with a flat membrane. So the results may not be quite so accurate in an average, though much care was taken to obtain a really stationary condition by extended observation and repeated renewal of the solution before definite readings were made. The results are plotted in Fig. 5.

Although the transfer number of Cl is low in any case, especially in low concentration ranges, still the difference can be plainly seen in the different alkali chlorides. The experiments with HCl were not carried out up to the same concentration as in the other electrolytes on account of doubt as to whether liquid junction potentials might be insufficiently abolished. It should be borne in mind that an error of 1 millivolt, according to the particular circumstances, may have a great influence on the evaluated transfer number. That is also the reason why these figures are not utilized in further computations

TABLE I.

Celloidin Membrane, Bag Form.

Change of the P.D. *with the Change of the Concentration Range. The Ratio of Concentration of Each Chain is Always 1:2. All Experiments with the Same Membrane.*

KCl solutions	P.D.				
	1st series of experiments (22°C.)	2nd series (22°C.)	3rd series (20.5–22°C.)	Average	r_{Cl}
	mv.	mv.	mv.	mv.	mv.
M/400 : M/200		17.8	17.7	17.7	0
M/200 : M/100	15.3		17.8	16.6	0.040
M/100 : M/50	14.7		16.2	15.5	0.059
M/50 : M/25			15.3	15.3	0.065
M/25 : M/125			12.5	12.5	0.145
M/20 : M/10	13.2	13.0	12.9	13.0	0.130
M/12.5 : M/6.25			9.5	9.5	0.230
M/6.25 : M/3.125			5.5	5.5	0.309
M/2 : M/1	4.8	3.9	3.8–3.1 (Aver. = 3.4)	4.0	0.386

Theoretical maximum value at 22° = 17.6 millivolts.

but only shown in the diagram where the interpolation curves, obtained a little arbitrarily, give an idea about the differences. The order of the transfer number of Cl in the different chlorides is the same as in aqueous solutions; the absolute values, however, strongly diminished in comparison with the latter, and the fact that the transfer number depends largely on the concentration, is obvious for all of these electrolytes, whereas in free aqueous solutions the influence of concentration is known to be small. Further discussion must be delayed.

TABLE II.

Celloidin Membrane, Flat Form.

Concentrations of KCl	Temperature	P. D.	r_{Cl}
		mv.	
N/1 : N/2	18.5°	3.5	.399
N/2 : N/4	18.5°	5.45	.343
N/4 : N/8	19.5°	9.55	.226
N/8 : N/16	19.5°	11.75	.163
N/16 : N/32	19.5°	14.5	.085
N/32 : N/64	20.5°	15.4	.060
N/64 : N/128	20.5°	16.35	.033

TABLE III.

Celloidin Membrane, Bag Form.

Temperature 20° ± 0.5°C. Theoretical Maximum Value of a Concentration Chain for This Temperature: 17.4 Millivolts.

	Concentration range	P. D.	Transfer Number of Cl
	M	mv.	
LiCl	.01–.02	15.1	.066
	.02–.04	13.4	.115
	.04–.08	11.4	.173
	.08–.16	8.3	.262
	.16–.32	5.2	.351
NaCl	.01–.02	15.5	.055
	.02–.04	14.8	.075
	.04–.08	13.5	.112
	.08–.16	10.5	.198
	.16–.32	6.2	.322
KCl	.01–.02	16.05	.026
	.02–.04	15.4	.058
	.04–.08	13.6	.109
	.08–.16	10.8	.190
	.16–.32	7.3	.290
HCl	.01–.02	17.2	.006
	.02–.04	17.1	.009
	.04–.08	15.65	.050
	.08–.16	15.5	.055

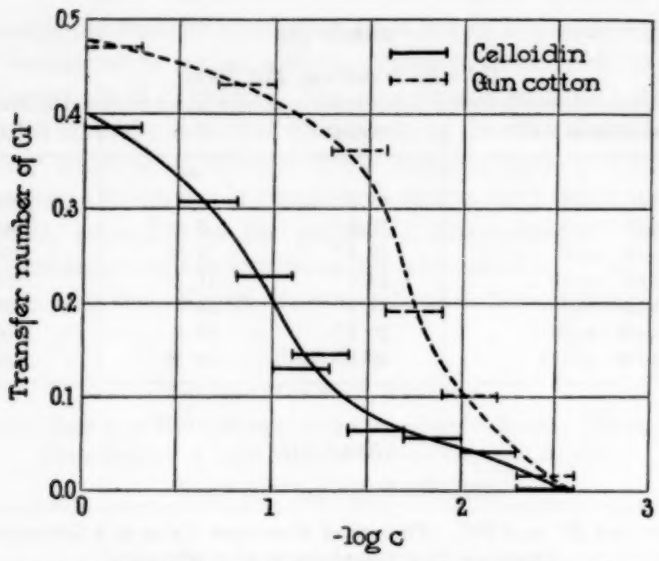

FIG. 3. Magnitude of the transfer number for Cl through the different concentration ranges with two membranes of the bag type.

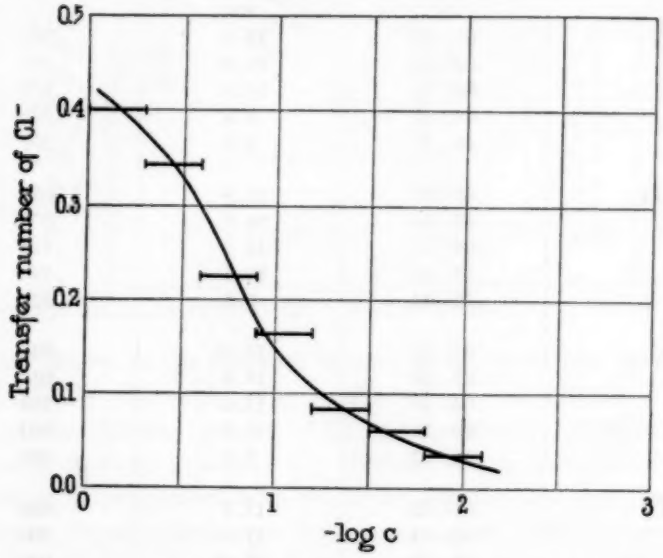

FIG. 4. Magnitude of the transfer number for Cl through the different concentration ranges with a collodion membrane of the flat bell jar type.

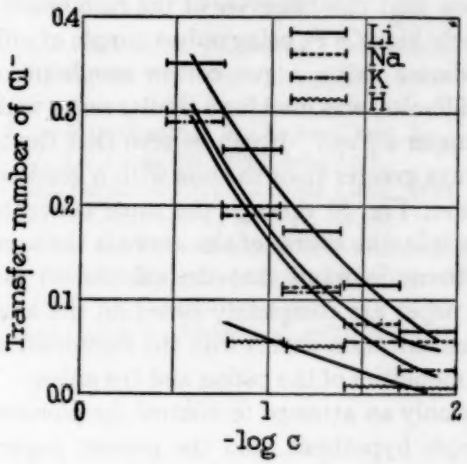

FIG. 5. Concentration chain curves showing the transfer numbers for Cl in the form of chlorides of different cations.

TABLE IV.

Gun Cotton Membrane, Bag Form.

Temperature 26.5°C. Maximum Value Calculated 17.7 Millivolts

KCl solutions	P. D.	f_{Cl} calculated
	mv.	
M/1 : M/2	1.10	0.47
M/5 : M/10	2.60	0.43
M/10 : M/20	4.05	0.40
M/20 : M/40	5.55	0.36
M/40 : M/80	10.90	0.19
M/80 : M/160	14.25	0.10
M/160: M/320	17.1	0.017
M/10 : M/100 (that is : *Co P*)	21.4*	0.306†

* 2 days ago 23.0.

† Theoretical maximum value for this chain = 55 millivolts. Corresponding to the value of the interpolation curve for a concentration = M/40.

In order to show that this behavior of the membrane is connected with their relatively high *Co P*, being only a couple of millivolts below the possible maximum value, a gun cotton membrane with a lower *Co P* (21 to 23 millivolts) was used for a similar series with KCl (Table IV and Fig. 3, upper curve). It can be seen that the transfer number of Cl is always greater than the one with a good celloidin membrane (lower curve, Fig. 3) through the same concentration range. However, in principle, the course of the curve is the same.

It should be borne in mind that the calculation of the transfer numbers in this paper are completely based on the assumption that the P.D. of the concentration chains with the membrane are due to the difference of the mobilities of the cation and the anion. This assumption was till now only an attempt to control the phenomena in membranes by a simple hypothesis, and the present paper shows some consequences of this hypothesis. Subsequent papers will have to deal with the problem of whether this hypothesis is sufficient or requires an addition or correction.

SUMMARY.

The ionic transfer number in an electrolyte solution in the pores of a narrow pored collodion membrane depends much more on the concentration than it does in a free aqueous solution. The potential difference of two solutions of the same electrolyte in different concentration depends largely on the concentration range. The ratio of the concentrations on the two sides was always 1:2 in the experiments; the concentration range was varied. It is shown that the transfer number of Cl, calculated from the P.D. measured, is very small in dilute solution (down to .02 and less in some cases), whereas it approaches the value .5 holding for free aqueous solutions when the concentration range is raised. The differences for the transfer number of Cl, according to the cation (H, K, Na, Li), can be recognized and show the same order as in free aqueous solution. But even in LiCl, where in an ordinary aqueous solution the transfer number of Cl is always $> .5$, this number is very low in the case of the membrane (*e.g.* $< .05$ in .01 M solution).

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STUDIES ON THE PERMEABILITY OF MEMBRANES.

III. Electric Transfer Experiments with the Dried Collodion Membrane.

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1. INTRODUCTION.

In a previous paper (1) an indirect method was described for determining the transfer number of an electrolyte in the dried collodion membrane. To furnish additional evidence in favor of the theory, it has seemed desirable to determine the transfer number by direct transfer experiments with an electric current. For a long time it has been known that the transfer number might be altered by a membrane. A number of investigators had tried to make use of various types of membranes to separate the different regions of the transfer apparatus and thereby avoid mechanical mixture. But as soon as it was recognized that the transfer number might be changed by the membrane, the method was abandoned. In the present investigations, however, this change of the transfer number is the very object of the investigations.

The alteration of transfer numbers by membranes observed by the earlier investigators is discussed by Hittorf (2), who found that only certain membranes, in which what he called the "Schlierenphænomen" could be observed, were able to cause any change. This phenomenon can sometimes be observed when two equal solutions are separated by a membrane and an electric current passed across the

* This investigation was begun by Dr. Yamatori while working in my laboratory in Nagoya, Japan. Due to lack of time he was unable to complete the experiments, but the Baltimore authors are greatly indebted to him for preliminary work in which the difficulties associated with these transfer experiments were recognized and overcome.

membrane in such a way that electroendosmosis occurs. Normally in the phenomenon of electroendosmosis the solution as a whole is forced unchanged through the membrane in the direction of the positive current. But in some cases (especially with membranes of gelatin and dried animal intestine and with electrolytes such as $CdCl_2$, $CaCl_2$, and HCl, but never with the usual uni-univalent neutral salts) the solution was separated into two different parts by the electroendosmosis. The solution coming through the membrane in the direction of the positive current was of a lower concentration than the original solution and that going in the opposite direction of higher concentration. The two layers could be distinguished with the naked eye because of refractive differences and even better with Töpler's "Schlierenapparat" (3). According to the contention of Hittorf it was only in those membranes which gave rise to this "Schlierenphænomen" that the transfer number differed from the one determined without a membrane. When the phenomenon was absent he believed that the membrane had no influence on the transfer number. Among the membranes showing the "Schlierenphænomen" Hittorf gives brief mention to the collodion membrane. His collodion membranes were in all probability of the dried type used in the present investigations, a fact which can be recognized because of the high electrical resistance which he attributed to them. The more common permeable collodion membranes of the present day when in contact with an electrolyte solution do not offer any conspicuous resistance to the passage of a current. Unfortunately Hittorf's comments on these changes of the transfer numbers are exceedingly brief and the change produced by collodion membranes was not determined. Relatively the observed alterations were small and just great enough to justify the claim that the membrane marred somewhat the accuracy of a transfer number. They did not approach in degree the changes of transfer number by membranes of dried collodion reported in our last paper nor was any note made of dependence on concentration.

Related to the Hittorf phenomenon is the phenomenon described by Bethe and Toropoff (4) which may be regarded as a special case of the more general Hittorf phenomenon. These authors described a sort of hydrolysis brought about by a membrane. When a membrane is interposed between two equal and very dilute solutions of a neutral salt and an electric current allowed to pass, an acid reaction

is brought about immediately adjacent to one side of the membrane and an alkaline on the other. This phenomenon is a special case of the Hittorf phenomenon insofar as a change of concentration of certain ions, namely H^+ and OH^-, is brought about by the membrane. This phenomenon can be observed in an appreciable degree only in salt solutions of such a high dilution that the ions of water participate appreciably in conducting the current within the membrane. As will be shown later this effect is small enough in our experiments to be neglected for the computation of the transfer numbers of the ions of the neutral salt.

The Hittorf effect is likewise closely associated with a phenomenon of electric transfer described by Nernst and Riesenfeld (5). When two equal aqueous solutions of an electrolyte are separated by a third conducting layer which contains the same electrolyte but is not miscible with water and an electric current is allowed to pass through the system, at times the electrolyte becomes concentrated on one side of the third layer and diluted on the other. Nernst and Riesenfeld discerned that this effect must be the result of a difference in the transfer number of the electrolyte in the aqueous and non-aqueous solutions and showed how the transfer numbers could be calculated from such experiments. Riesenfeld (5) made a number of these determinations, using such systems as water-phenol-water, containing electrolytes like KI or KCl in equilibrium throughout the system. The transfer numbers for the cation with KCl were as high as 0.8. He then attempted to determine these transfer numbers by another method; *i.e.*, by measuring the E. M. F. of concentration chains, but was unable to confirm the values obtained in the transfer experiments, the results of the second method giving a figure close to 0.64. A solution of these contradictory results was not attempted by Riesenfeld, nor has anyone undertaken a renewed investigation as far as we know. The membrane used by Riesenfeld differed from ours in being a homogeneous phase working as a solvent for the electrolyte as opposed to our dried collodion membrane which in all probability acts as a sieve. Nevertheless, this phenomenon of Nernst and Riesenfeld[1] observed in homogeneous membranes as well as the

[1] Only a brief discussion of this work by Nernst and Riesenfeld has been inserted here because it is not directly concerned with the properties of porous membranes. A complete review must be left for a more suitable occasion.

phenomenon of Hittorf observed in porous membranes can both be reduced to a difference of transfer numbers of the electrolyte within and without the membrane.

Our own experiments deal with the dried collodion membrane in which the determination of a transfer number is simplified in one respect though complicated in another. It is simpler because the error introduced by spontaneous diffusion, convection, and mechanical stirring is small and usually may be neglected. Separation of the different parts of the fluid is accomplished by the membrane. On the other hand a new source of error is introduced. At times the initial properties of a membrane may be markedly altered by the action of a strong electric current. These changes are partly reversible and partly irreversible; *i.e.*, permanent even after the electric current has been stopped. This action of an electric current on a membrane may be described and a method of obviating the difficulties involved pointed out.

2. The Behavior of the Dried Collodion Membrane in the Electric Current.

If two equal solutions of some electrolyte, *e.g.* Na_2SO_4 in 0.1 or 0.01 M concentration, are separated by a bag of completely dried collodion and a rather strong electric current (0.1–1 ampere) is allowed to pass between a platinum electrode inside and another outside the bag, after a certain time a sharp cracking noise will be heard, such as is produced by a spark breaking through an insulator. The noise soon becomes a permanent rattle and on inspection one can see a small leak which is apparent because of a rapid streaming of the liquid through it from one side of the membrane to the other. The stream is visible because of a difference of light refraction. When the leaks are of microscopic size the holes cannot be seen and are recognized only by this phenomenon of refraction. Gradually the leak becomes larger and a macroscopic hole can be seen. Simultaneously with the formation of a leak the intensity of the electric current begins to increase rapidly (unless diminished by the application of an external resistance) due, of course, to the increased conductivity through the hole in the membrane.

The description of this phenomenon as given is far from being complete, and must be studied more thoroughly. However, for the

present purposes it is sufficient to point out that the electric current exerts some influence on the pores of the membranes. Only when the voltage is high will the current strength be sufficiently great to cause an actual break in the membrane. However, when the voltage is lower, the same influence may be present though not great enough to produce a rupture. This supposition is rendered likely by the observation that the *Co P* of a membrane may be much lower after than before the weak current was passed. For example, one bag membrane with a *Co P* of 50 millivolts between 0.1 and 0.01 M KCl solutions, showed a P.D. of only 30–40 millivolts after the passage of the current. As a special procedure is necessary for measuring the *Co P* of membranes after the application of an electric current, a description of this procedure will be inserted here.

When the ordinary dried collodion membrane is placed between two KCl solutions of the same concentration, there is, of course, no P.D. However, after a membrane has been subjected to the passage of an electric current this may no longer be true. A P.D. is established even between two equal solutions. Evidently some kind of polarization has taken place. The distribution of the electrolyte ions within the membrane is no longer homogeneous. One side has become more concentrated, the other more dilute, and hence the P.D. The P.D. tends to fall but the fall is sometimes very slow especially when the membrane has been washed in distilled water. Even after a day of such washing a part of the polarization potential may still remain when the membrane is placed between two 0.01 M KCl solutions. However, it can quickly be diminished by washing with a stronger salt solution instead of pure water. When after washing with water some millivolts are established between two 0.01 M KCl solutions, the P.D. between two 0.1 M KCl solutions is always much smaller. When the membrane is allowed to stand for some minutes in the stronger solution and then replaced in the weaker one, the P.D. will have been definitely diminished. In this way one can depolarize a membrane completely, so that no P.D. will be established between two 0.01 M KCl solutions. Not until this behavior of the membrane has been reached can the P.D. between two *different* KCl solutions (0.1 and 0.01 M) separated by the membrane be measured with any reasonable meaning. With any membrane which has been subjected

to the passage of an electric current the absence of polarization must be strictly proven before its *Co P* is measured. When the current has been weak (<0.1 milliampere) and the time of exposure short, the polarization potential is usually small and easily overcome.

It is obvious that the permeability of a membrane will change when the pores have been dilated by the electric current. Just as the *Co P* is diminished, so will the difference in behavior between cations and anions be decreased. It is for this reason that in our first experiments we did not succeed in observing transfer numbers for the anion as low as had been expected. To be sure a transfer number of the anion with KCl close to 0.5 was never found. All experiments tended to confirm a diminished velocity of the anion in the membrane but the diminution was not great enough and not regular enough in the different experiments. When the difficulties had finally been recognized, it became possible to obtain very low transfer numbers for the anion. Those experiments were selected in which the *Co P* of the membrane turned out not to have been altered by the current. To insure this it was found necessary to apply only weak currents for a short time. As a consequence the quantity of ions transferred was very small and chemical analyses of a high degree of accuracy were not possible. Nevertheless by a slight modification of the procedure, to be described presently, the results of the chemical analyses were in excellent agreement and entirely sufficient for the purpose even though the limits of error were somewhat higher than under more favorable conditions.

Since the development of flat membranes of the type described in a previous paper (1), the difficulties due to alteration of the membrane by the current have been largely abolished. The properties of these membranes, as estimated by the *Co P*, have remained unchanged to the present time, a period of more than 4 months, even though used almost daily for transfer experiments. In the future there should be no difficulty in performing transfer experiments with collodion membranes.

3. Arrangements of Transfer Experiments.

The use of a dried collodion membrane to separate the different solutions in an electric transfer experiment is simple and convenient

when compared with the usual transfer experiments made in electrochemistry. The most simple arrangement is to separate two equal solutions of an electrolyte by the membrane, allow the current to flow, and then determine the change of concentration in the two solutions. This method, however, could not be used because the quantity of current necessary to change the concentrations sufficiently for satisfactory chemical analysis was large and even the most resistant membranes would eventually have been injured, especially when used for a long comparative series of experiments. Fortunately it was possible to modify the procedure so as to furnish better facilities for chemical analysis. Solutions of KNO_3 and NaCl of equal molarity were separated by the membrane, platinum electrodes introduced into each solution, and a current allowed to pass in the direction from KNO_3 to NaCl. In the very beginning of the experiment, before the circuit had been closed, the membrane would contain the ions of both of these two electrolytes. However, within a few moments after the current had been started, the Na^+ and NO_3^- would have been expelled from the membrane, and the current within the membrane conducted entirely by the K^+ and Cl^- ions migrating in opposite directions. The current was furnished by storage cells and regulated by a finely graduated ballast resistance. As a result of polarization taking place at the platinum electrodes the current always tends to fall during the first minutes of the experiment but it is easy to maintain a current of constant intensity (at least within $\frac{1}{2}$ to 1 per cent of the total value) by watching a sensitive milliammeter and varying the amount of ballast resistance by hand. When the membrane itself does not cause trouble due to injury by the current, the necessary change in the applied resistance is quick only in the first few minutes, then a gradual and uniform regulation is required, and finally no essential variation of the resistance is necessary even for a long period.

In the first experiments membranes of the bag form were employed. The electrode inside the bag was a platinum spiral; for the outside electrode a large platinum net bent in such a way as to surround at least half the circumference of the bag was used. It was always kept at a reasonable distance from the membrane (2–3 cm.). The following observations were made.

1. The total quantity of current (calculated from product of milliamperes and seconds). From this the total amount of ionic transfer could be computed from the formula

1 milliampere × 1 minute = 0.000622 milli-equivalents of ions.

The accuracy of this method was checked several times by the use of Herroun's iodine coulometer as recommended by Ostwald and Luther[2] and found to be excellent. With the small amounts of current used in the experiments it proved more satisfactory and convenient than any form of coulometer.

2. The quantity of potassium transferred into the NaCl solution.

3. The quantity of chlorine transferred into the KNO_3 solution.[3]

4. In some cases the amount of acid developed in the anode chamber.

In these experiments the assumption is necessary that practically all of the current is carried by the ions of the electrolyte and that the water ions do not participate to an appreciable extent even within the membrane. Under this assumption the quantity of H^+ ions developed in the anode chamber must correspond to the total quantity of current (milliamperes × seconds) and be equal to the sum of the K and Cl transferred. If it were true that a portion of the current was carried by the water ions this equality would no longer hold for the change of acidity would take place not only at the platinum electrodes but also at the membrane (Bethe-Toropoff effect).[4] This

[2] Ostwald, W., and Luther, R., Hand- und Hilfsbuch zur Ausfuhrung physikochemischer Messungen, Leipsic, 1925, 4th edition, 569.

[3] A slight modification of the procedure for determining Cl described in the first paper of this series seemed useful. The solution, which as a rule was acid as a result of the electric transfer, was slightly alkalinized by KOH to a slight pink with phenolphthalein, evaporated almost to dryness, acidulated with just sufficient N/10 acetic acid to decolorize the phenolphthalein, then treated with potassium chromate as before described. Approaching the end-point of the titration with N/100 $AgNO_3$, after each drop of silver solution the liquid was centrifugalized. Under these conditions the first trace of a precipitate of silver chromate could be recognized as a brownish layer above the white AgCl. The increased labor involved in the centrifuging brings increased accuracy. 1 drop of excess of $AgNO_3$ N/100 results in a striking brown layer.

[4] The most important paper of Bethe and Toropoff (4) belongs to the same class of phenomena as the Hittorf effect. They are concerned only with the changes in concentrations of H^+ and OH^- ions on both sides of a membrane

effect can be of appreciable magnitude only in very dilute solutions. In our experiments the agreement obtained between total current and sum of migrated electrolyte ions was close enough to indicate that neglecting the Bethe effect introduced no error of greater magnitude than that involved in the chemical analyses.

4. THE EXPERIMENTAL RESULTS.

A. Check of the Method.

The most frequently used arrangement of apparatus in the experiments was as follows:

Large platinum net (anode)	0.2 M KNO_3	Membrane	0.2 M NaCl	Large platinum spiral (cathode)

The intensity of the current was measured with a milliammeter and regulated with a variable resistance. The first problem was to check the soundness of the method by proving that the amount of chlorine transferred in one direction and K in the other was equivalent to the total transfer as estimated from the coulombs applied. It is obvious that no K can be lost during the experiment but it is just possible that a portion of the Cl entering the KNO_3 solution might be oxidized at the electrode and removed from the solution. In view of the small number of Cl^- ions in the anode chamber relative to the NO_3^- ions, even at the end of the experiment, it does not seem likely that any appreciable amount could be set free at the electrode. Nevertheless, it seems desirable to prove by experiment that the small quantities of Cl found are in reality due to the fact that Cl plays only a minor rôle in carrying the current and not to a loss of Cl.

Table I lists the results of two experiments in which the total ionic equivalent calculated from the coulombs passed is compared with the sum of K and Cl found by analysis. The agreement is very good in view of the analytical difficulties involved and quite sufficient to prove that no appreciable amount of Cl is lost. It also shows that any current transported by water ions (Bethe-Toropoff effect) is too

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brought about by an electric current. This change of pH which we called the "Bethe-Toporoff effect" occurs in an appreciable amount only in very dilute electrolyte solutions.

small to produce a measurable error. Having shown that this agreement holds we are justified in using analytical figures for Cl in experiments in which the amount of K or analogous cation was not or could not be determined and the total ionic transfer estimated only from the coulombs passed.

TABLE I.

Potassium Titrations (1/3 of Total Quantity Taken for Analysis).

Experiment No.	N/50 Na oxalate cc. used	$KMnO_4$ solution	Corrected by factor to N/100	N/100 $KMnO_4$ used	Millimols K	Total K found	Average corrected for K in reagents	Total transfer	
								According to the analyses for K and Cl	According to the coulombs passed
135 (1)	1.40	4.12	4.34	2.94	.01070	.03210	.03186	.03744	.0373
135 (2)	.70	3.86	4.07	3.37	.01226	.03678			
136 (1)	.80	3.86	4.07	3.27	.01190	.03570	.03424	.03982	.0373
136 (2)	.60	3.88	4.08	3.48	.01265	.03795			

Check analyses to determine necessary correction for amount of potassium in NaCl used. .035 millimols K added to 50 cc. N/5 NaCl (amount used in above experiments). 1/3 of total taken for analyses.

1	.60	4.11	4.33	3.73	.01357	.04071	Average = .03758
2	1.10	4.11	4.33	3.23	.01175	.03525	Added K present = .03500
3	.90	4.06	4.27	3.37	.01226	.03678	K in reagent = .00258

Table to show accuracy of agreement between milli-equivalents calculated from the current passed and actual transfer of ions as determined by analysis. N/5 KNO_3 solution surrounding positive electrode. N/5 NaCl solution surrounding negative electrode. Solutions separated by C_1 collodion membrane. Current strength = 0.01 ampere for 6 minutes. Total milli-equivalents of current = .03732 (short time used to avoid error due to diffusion).

In four identical experiments Cl^- transferred was .0054; .0054; .0063; and .0052 millimols, respectively. Average figure of .00558 used in calculation of total transfers.

In some of the later protocols similar checks will be found but special care was taken in the experiments of Table I. In these experiments the solutions were in contact with the membrane for less than 7 minutes (total current time 6 minutes) and the time was too short for a measurable error due to spontaneous diffusion. In other experiments where the current intensity was smaller and the current

time was longer, a small correction was necessary for K spontaneously diffused, disturbing somewhat the accuracy of agreement. In none of the experiments could we show that a significant amount of Cl migrated by spontaneous diffusion. Therefore this factor can play no part in disturbing the accuracy of the transfer number for Cl when it is calculated from the Cl found by analysis and the total transfer estimated from the current applied.

B. Protocols of Transfer Experiments.

1. Bag Membrane 31.

a. Characteristics of the Membrane.—*Co P* before the transfer experiment = 52.0 millivolts. After the experiment the membrane was washed in distilled water for 3 days and then the following successive P.D. measurements made.

1. 0.01 M KCl on both sides of membrane.....2.3 millivolts.
2. 0.1 M KCl on both sides of membrane.....0.0 millivolt.
3. 0.01 M KCl on both sides of membrane..0.0 millivolt. (This reading made after solutions had been in contact with membrane for 30 minutes.)
4. *Co P*.....47.1 millivolts.

b. The Transfer Experiment.—Outside solution 0.1 M KNO_3; inside solution 0.1 M NaCl; current strength 0.500 milliamperes; time 70 minutes.

K^+ transferred (analysis of NaCl solution).....0.0182 milli-equivalents.
Cl^- transferred (analysis of KNO_3 solution).....0.0045 milli-equivalents.
Total observed transfer ($K^+ + Cl^-$).....0.0227 milli-equivalents.
Total expected transfer.....0.0218 milli-equivalent.

$$t_{Cl^-} = \frac{Cl^-}{K^+ + Cl^-} = 0.20$$

c. Agreement with Transfer Number Determined by the Concentration Chain Method.—After the transfer experiment P.D. measurements were made of the membrane with the following solutions and the transfer numbers calculated according to the method previously described.

0.05 M KCl : 0.1 M KCl — P.D. = 12.8 mv. at 24°C. $t^- = 0.14$
0.1 M KCl : 0.2 M KCl — P.D. = 9.8 mv. at 24°C. $t^- = 0.22$

The transfer number observed in the transfer experiment (0.20) lies between the value of the two average transfer numbers calculated from the concentration chains; *i.e.*, $0.14 < 0.20 < 0.22$.

2. Bag Membrane 100.

a. Characteristics of the Membrane.—*Co P* before the transfer experiment = 52.5 millivolts. Following the experiment and after abolition of the polarization potential *Co P* was only 42.3 millivolts.

b. The Transfer Experiment.—Outside solution 0.01 M KNO_3; inside solution 0.01 M NaCl; current strength 0.125 milliampere; time 150 minutes.

K^+ transferred	0.0095 milli-equivalents.
Cl^- transferred	0.0009 milli-equivalents.
Total observed transfer ($K^+ + Cl^-$)	0.0104 milli-equivalents.
Total expected transfer	0.0117 milli-equivalents.

$$t_{Cl^-} = \frac{Cl^-}{K^+ + Cl^-} = 0.08$$

c. Agreement with Transfer Number Determined by the Concentration Chain Method.—Following the experiment the P.D. of the membrane was measured in the following concentration chains.

KCl 0.005 M : 0.01 M — P.D. = 16.3 mv. at 24°C. $t^- = 0.039$
KCl 0.01 M : 0.02 M — P.D. = 13.2 mv. at 24°C. $t^- = 0.126$

The value of t^- as observed in the transfer experiment lies between the values of the two average transfer numbers calculated from the concentration chains; *i.e.*, $0.039 < 0.08 < 0.126$.

3. *Bag Membrane 47.*

a. Characteristics of the Membrane.—*Co P* before the transfer experiment = 54.3 millivolts. *Co P* after the experiment and after abolition of polarization potential = 50.3 millivolts.

b. The Transfer Experiment.—Outside solution 0.01 M KNO_3; inside solution 0.01 M NaCl; current strength 0.125 milliampere; time 150 minutes.

K^+ transferred	0.0118 milli-equivalents.
Cl^- transferred	<0.0016 milli-equivalents.[5]
Total observed transfer ($K^+ + Cl^-$)	<0.0134 milli-equivalents.
Total expected transfer	0.0117 milli-equivalents.

$$t_{Cl^-} = \frac{Cl^-}{K^+ + Cl^-} < 0.14$$

c. Agreement with Transfer Number Determined by the Concentration Chain Method.—Following the experiment the P.D. of the membrane was measured in the following concentration chains.

KCl 0.005 M : 0.01 M — P.D. = 16.1 mv. at 24°C. $t^- = 0.046$
KCl 0.01 M : 0.02 M — P.D. = 15.1 mv. at 24°C. $t^- = 0.074$

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[5] The amount of Cl^- here represented corresponds to 4 drops of the 0.01 M $AgNO_3$ used in titrating and is too small for accuracy. The quantity recorded must be considered as a maximum value and is probably too high.

The value of t^- as observed in the transfer experiment is slightly greater than that estimated from the concentration chains but of the same order of magnitude and as such, in view of the analytical difficulties, in accordance with expectations.

4. Flat Bell Jar Membrane C_1.

The results of a long series of transfer experiments with this membrane have been arranged in Table II.

5. DISCUSSION.

The transfer experiments reported in this paper are confirmatory of the results obtained by the indirect method previously described (1), namely that in the membrane the transfer number of the anion is much smaller than that of the cation and that this difference is the most marked in the more dilute solutions. The finding of a membrane with such durable properties as the type with which the experiments of Table II were performed made it possibe to carry out a long series of experiments in which the effect of varying the cation and the concentrations could be observed. Here again it will be seen that the transfer number of Cl^- in solutions of equal concentration but with different cations is in the order Li > Na > K > H. Moreover, the transfer numbers regularly become greater as the concentration of the electrolytes is increased. This membrane was also used in a series of 2:1 concentration chain experiments with KCl (1) and it is possible to compare the transfer numbers in the different ranges of concentration as estimated by this method with those actually found in the transfer experiments. The result is shown in Fig. 1. On the whole the agreement is good. Whether the minor discrepancies are the result of some systematic and unrecognized error or due to an additional unknown factor not taken into account in the theory is not clear. In this connection it is desirable to call attention to an observation which was made during the series of experiments and for which no satisfactory explanation is as yet apparent. When the membrane was used in a short space of time for several successive transfer experiments, almost invariably it was found that the transfer number for Cl^- calculated from the results of the first experiment was somewhat higher than the transfer numbers computed from the succeeding experiments. Had the reverse

TABLE II.

Experiment No.	Solution in anode compartment	Solution in cathode compartment	Current strength	Time	Calculated total transfer	Millimols K found	Millimols Cl found	Total transfer found	Transfer No. Cl^-
			milliamperes	*min.*	*milli-equivalents*				$\frac{Cl^-}{\text{calculated total transfer}}$
A	M/50 KNO_3	M/50 NaCl	2	15	.01866	.01812	.0023	.02042	0.123
2	M/50 KNO_3	M/50 NaCl	2	30	.03732		.0045		0.121
3	M/50 KNO_3	M/50 NaCl	2	30	.03732		.0030		0.080
8	M/50 KNO_3	M/50 KCl	2	30	.03732		.0039		0.104
143	M/50 KNO_3	M/50 KCl	6	10	.03732		.0045		0.121
144	M/50 KNO_3	M/50 KCl	6	10	.03732		.0020		0.054
145	M/50 KNO_3	M/50 KCl	6	20	.07464		.0045		0.060
30	M/10 KNO_3	M/10 NaCl	2	35	.04354	.0402	.0043	.04452	0.095
31	M/10 KNO_3	M/10 NaCl	2	30	.03732	.0405	.0035	.04401	0.094
37	M/5 KNO_3	M/5 NaCl	2	30	.03732	.0393	.0043	.04362	0.116
39	M/5 KNO_3	M/5 KCl	2	30	.03732		.0059		0.157
40	M/5 KNO_3	M/5 KCl	2	32	.03981		.0048		0.122
42	M/5 KNO_3	M/5 NaCl	4	15	.03732	.0360	.0053	.04135	0.143
43	M/5 KNO_3	M/5 NaCl	4	15	.03732	.0374	.0047	.04215	0.127
133	M/5 KNO_3	M/5 NaCl	10	6	.03732	.0414	.0054	.04680	0.145
134	M/5 KNO_3	M/5 NaCl	10	6	.03732	.0388	.0054	.04420	0.145
135	M/5 KNO_3	M/5 NaCl	10	6	.03732	.0319	.0063	.03816	0.169
136	M/5 KNO_3	M/5 NaCl	10	6	.03732	.0342	.0052	.03944	0.139
146	M/5 KNO_3	M/5 KCl	6	20	.07464		.0175		0.234
147	M/5 KNO_3	M/5 KCl	6	20	.07464		.0100		0.134
148	M/5 KNO_3	M/5 KCl	6	20	.07464		.0085		0.114
34	M/2 KNO_3	M/2 NaCl	2	30	.03732		.0076		0.205

35	M/2 KNO_3	M/2 NaCl	2	30	.03732		.0068	0.183
131	M/2 KNO_3	M/2 NaCl	2	30	.03732		.0077	0.206
132	M/2 KNO_3	M/2 NaCl	2	30	.03732		.0099	0.265
47	M/1 KNO_3	M/1 NaCl	4	15	.03732		.0108	0.290
139	M/1 KNO_3	M/1 KCl	6	10	.03732		.0150	0.402
140	M/1 KNO_3	M/1 KCl	6	11	.04105		.0164	0.400
141	M/1 KNO_3	M/1 KCl	6	10	.03732		.0146	0.391
1	M/50 $NaNO_3$	M/50 NaCl	2	30	.03732		.0082	0.220
6	M/50 $NaNO_3$	M/50 NaCl	2	30	.03732		.0065	0.174
7	M/50 $NaNO_3$	M/50 NaCl	2	30	.03732		.0057	0.152
13	M/50 $NaNO_3$	M/50 NaCl	2	30	.03732		.0100	0.268
14	M/50 $NaNO_3$	M/50 NaCl	2	30	.03732		.0077	0.206
15	M/50 $LiNO_3$	M/50 NaCl	2	30	.03732		.0131	0.349
16	M/50 $LiNO_3$	M/50 LiCl	2	30	.03732		.0099	0.266
17	M/50 $LiNO_3$	M/50 LiCl	2	31	.03856		.0113	0.294
18	M/50 $LiNO_3$	M/50 LiCl	2	30	.03732		.0097	0.261
19	M/50 $LiNO_3$	M/50 LiCl	2	30	.03732		.0056	0.150
23	M/50 $LiNO_3$	M/50 LiCl	2	30	.03732		.0083	0.221
24	M/50 $LiNO_3$	M/50 LiCl	2	60	.07464		.0196	0.263
27	M/10 $LiNO_3$	M/10 LiCl	2	31	.03856		.0124	0.322
28	M/10 $LiNO_3$	M/10 LiCl	2	30	.03732		.0112	0.299
4	M/50 HNO_3	M/50 HCl	2	30	.03732		.0013	0.035
5	M/50 HNO_3	M/50 HCl	2	35	.04354		.0012	0.028
9	M/50 HNO_3	M/50 KCl	2	30	.03732		.0017	0.046

Tabulation of results in 46 electric transfer experiments performed with the same dried collodion membrane (C_1-flat type). Experiments were done during a period of 4 months, the constancy of the properties of the membrane being shown by frequent *Co P* determinations. *Co P* at beginning of experiments (9–28–26) 48.7 millivolts; (11–8–26) 48.5 millivolts; (12–11–26) 49.0 millivolts; at end of experiments (1–21–27) 49.0 millivolts.

been true and the later experiments given higher transfer numbers we should have supposed that the electroendosmosis had temporarily stretched the membrane pores, lowered its *Co P*, and increased its permeability for the anion. However, the observed findings were the opposite of that which might have been expected. It scarcely seems wise to attempt an explanation until more experimental data has been accumulated. However, the variations in the transfer numbers referred to here were of a minor degree and do not interfere

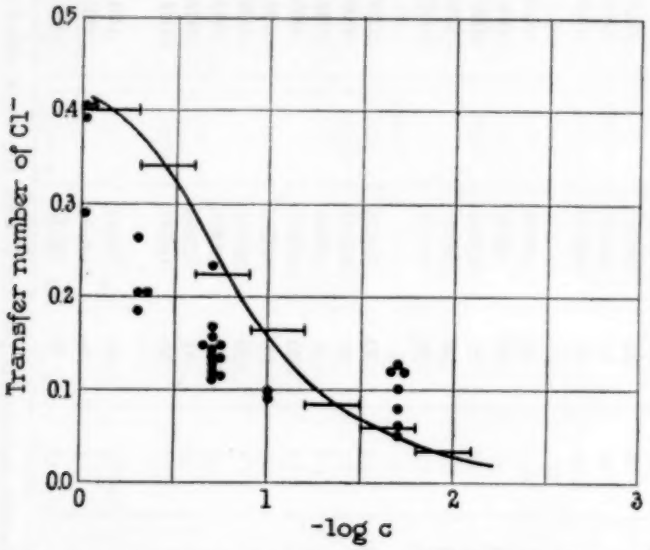

FIG. 1. Shows accuracy of agreement between chlorine transfer numbers with KCl as determined by the concentration chain method (continuous curve) and by electric transfer experiments (separate dots).

with their interpretation in the present communication. The transfer numbers determined in these experiments correspond fairly closely to those calculated indirectly from the concentration chains and furnish an additional reason for maintaining the theory set forth in connection with the latter method. We wish, however, to emphasize that the agreement between the two methods is not complete and that we believe that the range of variation is greater than the limits of error. Obviously the theory in its present state of development is insufficient to explain all data in a really quantitative way.

SUMMARY.

The transfer numbers of the ions of electrolytes in the dried collodion membrane, as determined in a previous paper indirectly from the E.M.F. of concentration chains, can also be determined directly by electrical transfer experiments. It is shown that the difficulties involved in such experiments can be overcome. The transfer numbers obtained by the two methods are in satisfactory agreement. The experimental results obtained in the transfer experiments furnish an additional argument in favor of maintaining the theory that the electromotive effects observed in varying concentrations of different electrolytes with the dried collodion membrane may be referred to differences in the mobilities of the anions and cations within the membrane. As was shown by the method of the previous paper, the transfer number depends largely on concentration. There are some minor discrepancies between the values of the transfer numbers obtained by the two methods which, as yet, cannot be completely explained.

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THE ACTIVATION OF STARFISH EGGS BY ACIDS.

II. The Action of Substituted Benzoic Acids and of Benzoic and Salicylic Acids as Influenced by Their Salts.

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INTRODUCTION.

In a recent paper[1] I described experiments indicating that the molecular rate of action of penetrating and parthenogenetically effective acids (fatty acids, carbonic acid, benzoic acid) is determined primarily by their strength as acids. In a pure solution of any single acid (within the range of concentrations effective in activation) the activation process within the egg proceeds at a rate which is closely proportional to the concentration of acid. If, comparing different acids, we consider solutions having as nearly as possible the same physiological effect, *e.g.*, causing complete activation in 10 minutes at 20°, we find a significant relationship: namely, the square root of the product of the dissociation constant into the concentration of acid appears nearly constant. Since the C_H shows a similar proportionality in solutions of weak acids in the absence of their salts ($C_H = \sqrt{K \times C_{acid}}$), this relationship might seem to indicate that the C_H of the external solution is the essential factor determining the rate of action. This, however, is shown not to be the case by the effects following the addition of the sodium salt of the acid to the solution. For example, the addition of .002 M Na acetate to the .002 M solution of acetic acid lowers the ionization of the acid—from *ca.* 10 per cent to *ca.* 1.5 per cent—thus bringing the C_H nearer to neutrality and correspondingly increasing the concentration of undissociated molecules. The fact that the rate of activation is *increased* by this addition, and to

[1] Lillie, R. S., *J. Gen. Physiol.*, 1925–27, viii, 339.

a degree (*ca.* 10 per cent) corresponding closely to the estimated increase in the undissociated molecules of acid, indicates that the essential factor in the activating effect is the concentration of the undissociated free acid in the external solution, and not the concentration of the ions of the acid. Apparently the undissociated acid molecules penetrate the egg rapidly and dissociate in its interior, serving there as a source of H ions; these then in some way induce activation, at a rate proportional to their concentration. The observed relationship between the strength of the acid and its activating effectiveness is thus explained. The rate of the activation reaction, within the appropriate range of C_H, would thus seem to be directly proportional to the C_H at the site of the activation reaction inside the egg, apparently in the cortical region of the latter.

Since this relation is an interesting one, I have continued these experiments during the past summer, using several additional acids. The number of acids available for this purpose is not large. Many apparently effective acids which cause typical membrane formation and early cleavage have a secondary toxic action and the eggs die before reaching the blastula stage. This is the case, for example, with caprylic acid and the three chloroacetic acids; and during the past summer the same was found true for phthalic acid. The membrane-forming action, taken alone, has a certain value as an index of the relative physiological effectiveness of acids; thus the relative rates of action of mono-, di-, and trichloroacetic acids as thus estimated are in the order of their dissociation constants;[1] but this test is a less satisfactory one than the production of blastulæ in a large proportion of eggs, and the latter index has been mainly relied upon in these experiments.

It seemed probable that the question of the relation between strength and activating efficiency could best be tested by using a series of acids varying in strength but similar in their constitution and presumably also in their ability to penetrate the egg. Since benzoic acid had proved highly effective as an activating agent in low concentrations (.00025 M to .001 M), experiments with a series of substituted benzoic acids were tried, including phthalic, salicylic, *o*-, *m*-, and *p*-chlorobenzoic, and *o*-, *m*-, and *p*-nitrobenzoic. These acids are relatively non-toxic in the low concentrations used, and have

a wide range of dissociation constants. The following are the values given in the Landolt-Börnstein Tables:[2]

TABLE I.

Acid	Dissociation constant
Benzoic	6×10^{-5}
Salicylic	1.06×10^{-3}
Phthalic (first constant)	1.26×10^{-3}
o-Chlorobenzoic	1.32×10^{-3}
m-Chlorobenzoic	1.55×10^{-4}
p-Chlorobenzoic	9.3×10^{-5}
o-Nitrobenzoic	6.3×10^{-3}
m-Nitrobenzoic	3.5×10^{-4}
p-Nitrobenzoic	4.0×10^{-4}

The method used was the same as in the previous series. The acids were dissolved in isotonic NaCl-$CaCl_2$ solution (100 volumes 0.52 M NaCl + 5 volumes 0.5 M $CaCl_2$). 100 cc. of a given solution of acid (at 20°) was added to 1 or 2 cc. of a dense suspension of the eggs in sea water, and at regular intervals (of 1 or 2 minutes) eggs were transferred in serial order to bowls containing sea water. Later the eggs were examined and the proportions developing to a swimming stage (blastula or gastrula) were determined.

Results with Different Acids.

The following summaries give the essential results obtained with pure solutions of the various acids.

Benzoic Acid.—The results previously obtained with this acid were confirmed. Using the concentrations, .0004 M, .0005 M, and .0006 M, the characteristic optimum exposures were, respectively, 12 to 16 minutes, 10 to 12 minutes, and *ca.*8 minutes. A large majority of eggs thus exposed (varying from 70 to 90 per cent in different experiments) formed blastulæ.

Phthalic Acid.—The concentrations used were .0002 M, .0003 M, .0004 M, and .0006 M, with exposures ranging from 2 to 20 minutes. This acid proved ineffective as an activating agent, apparently because of some secondary toxic action. It forms typical membranes, but the eggs break down before reaching

[2] Landolt, H., and Börnstein, R., Tabellen, Berlin, 5th edition, 1923, 1138 *et seq.*

swimming stages. The minimum exposures required to form membranes in 90 per cent (or more) of the eggs were (for the above four concentrations), respectively, 10, 6, 4, and 2+ minutes. The rate of membrane-forming action is thus closely proportional to concentration; it is, however, slower than that of benzoic acid, which in concentrations so low as .00025 M forms membranes in nearly all eggs with exposures of 2 to 3 minutes. This relatively gradual action of phthalic acid, as compared with the weaker benzoic acid, may be an indication of slower penetration, and has its parallel in the slowness of action of *o*-nitrobenzoic acid. The specific toxicity of the acid is an independent property, apparently analogous to that of oxalic acid.

Salicylic Acid.—In appropriate concentrations (.0002 M to .0006 M) this acid causes typical activation in a large proportion of eggs. Its molecular rate of action is between two and three times that of benzoic acid; its toxicity also is distinctly greater. An exposure of 2 minutes to the .0002 M solution is sufficient for membrane formation; exposures of 14 to 16 minutes (the optimum for .0002 M) cause most eggs to form blastulæ. Stronger solutions act more rapidly, but less favorably. Within the above range the rate of action is approximately proportional to concentration. The following is a summary of observations made during June, 1926, with good controls (Table II).

TABLE II.

Salicyclic Acid. Temperature 20°.

Concentration	Optimum exposures and percentages of eggs forming blastulæ
M	
.0002	(*7 series*) 16 min. (20–25 per cent); 14–16 min. (*ca.* 50 per cent); 13–15 min. (35–45 per cent); 16–18 min. (65–70 per cent); 12–14 min. (50–60 per cent); *ca.* 16 min. (70–80 per cent); 14 min. (35–45 per cent)
.0003	(*2 series*) 8–10 min. (30–40 per cent); 7–6 min. (20–30 per cent)
.0004	(*3 series*) 8–10 min. (5–10 per cent); 8–10 min. (30–40 per cent); 5–6 min. (25–30 per cent)
.0006	(*1 series*) 2–4 min. (few)

Chloro- and Nitrobenzoic Acids.—With the exception of *o*-nitrobenzoic acid, all the acids of this group activate more rapidly than benzoic acid. In the case of the meta- and para-acids the greater rate of action corresponds closely with that calculated from the dissociation constants. It is remarkable, however, that both ortho-acids, particularly *o*-nitrobenzoic acid, are much less effective than their very considerable strength as acids would lead us to expect. The following summaries give the chief observations with the single acids.

o-Chlorobenzoic Acid.—This acid is somewhat more rapid in its action than benzoic acid; its specific toxicity is also greater, and fewer eggs form blastulæ at

the optimum exposures. The following series (Table III) is typical (June 18, 1926).

m- and p-Chlorobenzoic Acids.—These acids are closely similar in their rates of action. On account of their slight solubility the concentrations used were limited to .00025 M and .00035 M for the *m*-acid, and .00025 M for the *p*-acid.[3] The following results are typical (Table IV).

All three chlorobenzoic acids act more rapidly than benzoic acid, but the ortho-acid is decidedly slower in its action than the other two, which are closely similar. This difference is illustrated in the series summarized in Table V, in which equimolecular solutions (.00025 M) of the four acids were compared in their action on the same lot of eggs.

TABLE III.

o-Chlorobenzoic Acid. Temperature 20°.

Concentration	Optimum exposures and percentages of blastulæ
M	
.0002	*Ca.* 34 min. (30–40 per cent)
.0003	22–26 min. (35–45 per cent)
.0004	12–14 min. (20–25 per cent)
.0005	*Ca.* 8 min. (20–25 per cent)

TABLE IV.

m- and p-Chlorobenzoic Acids. Temperature 20°.

Solution	Optimum exposures and percentages of blastulæ
M	
(*m*-Chlorobenzoic) .00025	(*1 series*) 7–8 min. (35–45 per cent)
.00035	(*1 series*) 3–4 min. (30–40 per cent)
(*p*-Chlorobenzoic) .00025	(*3 series*) 7–9 min. (25–35 per cent); 7–8 min. (30–40 per cent) 9–11 min. (65–75 per cent)

Ortho-chlorobenzoic acid, although the strongest of the three chloro-acids, acts more slowly than the meta- and para-acids and apparently is somewhat more toxic; of the two latter the meta-acid is somewhat the more effective. This unexpected behavior of the ortho-acid seems to indicate some difficulty in penetration, as in the analogous case of *o*-nitrobenzoic acid.

[3] The concentrations of the saturated solutions in distilled water were determined by titration with N/50 NaOH, using brom thymol blue as indicator. The solutions in isotonic NaCl-$CaCl_2$ were made by diluting the distilled water solution with a concentrated NaCl-$CaCl_2$ solution of a strength such that when the dilution was made the resulting solution was isotonic.

o-Nitrobenzoic Acid.—This acid, the strongest of the group, is somewhat surprisingly ineffective. Exposures up to 14 minutes, with the concentrations .0002 M, .00025 M, .0003 M, .0004 M, and .0005 M, gave only slight activation in any case. The longest exposure (14 minutes) to the .0005 M solution resulted in less than 1 per cent of blastulæ. The membrane-forming action is also gradual, indicating slow penetration; the exposures required to form membranes in 50

TABLE V.

Benzoic and Chlorobenzoic Acids. Temperature 20°.

Acid	Optimum exposures and percentages of blastulæ
.00025 M	
Benzoic	Longest exposure 24 min. (20–30 per cent); optimum not reached
o-Chlorobenzoic	Optimum not reached; *ca.* 5 per cent blastulæ at 12 min.
m-Chlorobenzoic	7–8 min. (35–45 per cent)
p-Chlorobenzoic	9–11 min. (65–75 per cent)

The exposures were 1 to 24 minutes for benzoic acid and 1 to 12 minutes for the other three acids; eggs were transferred to sea water at intervals of 1 minute.

TABLE VI.

m-Nitrobenzoic Acid. Temperature 20°.

Concentration	Optimum exposures and percentages of blastulæ
M	
.0001	(*1 series*) few membranes formed up to 30 minutes exposure
.0002	(*3 series*) 18–24 min. (40–50 per cent); 24–28 min. (*ca.* 50 per cent);
.00025	24–28 min. (*ca.* 50–60 per cent)
.0003	(*3 series*) 12–16 min. (30–40 per cent); 16–18 min. (40–50 per cent); *ca.* 6 min. (30–40 per cent)
.0004	(*5 series*) 10–12 min. (*ca.* 50 per cent); 8–12 min. (30–40 per cent); 8–10 min. (30–35 per cent); 5–7 min. (10–15 per cent); *ca.* 4 min. (25–35 per cent)
.0005	(*3 series*) *ca.* 6 min. (20–25 per cent); 5–6 min. (20–25 per cent); 4–5 min. (*ca.* 10 per cent)

per cent of eggs, with the concentrations .0002 M, .0003 M, and .0004 M, were, respectively, 7 to 9 minutes, 6 to 7 minutes, and 4 to 5 minutes. Membranes are formed by the .0002 M solutions of the other substituted acids and benzoic acid in 2 to 3 minutes.

m-Nitrobenzoic and p-Nitrobenzoic Acids.—A summary of the observations with these acids is given in Tables VI and VII. The two are closely similar in their action, with the *p*-acid slightly more rapid and slightly more toxic than the *m*-acid.

The differences between benzoic acid and the nitrobenzoic acids are brought out clearly in the series summarized in Table VIII, in which the four acids in equal concentrations (.00025 M) were used with the same lot of eggs.

It is clear that nitro-substitution in the meta- and para-positions greatly increases the effectiveness of benzoic acid. The same is true

TABLE VII.

p-Nitrobenzoic Acid. Temperature 20°.

Concentration	Optimum exposures and percentages of blastulæ
M	
.0002	(*1 series*) 16–18 min. (20–25 per cent)
.00025	(*1 series*) 9–10 min. (30–35 per cent)
.0003	(*3 series*) 8–12 min. (10–20 per cent); *ca.* 6 min. (15–25 per cent); 4–5 min. (25–30 per cent)
.0004	(*3 series*) 6–8 min. (10–20 per cent); 3–4 min. (15–20 per cent); 3–4 min. (20–25 per cent)
.0005	(*3 series*) 3–4 min. (15–20 per cent); 4–6 min. (5–10 per cent); *ca. 3* min. (15–20 per cent)

TABLE VIII.

Benzoic Acid and Nitrobenzoic Acids. Temperature 20° (July 26, 1926).

Acid	Optimum exposures and percentages of blastulæ
.00025 M	
Benzoic	30 min. + (50 per cent at 30 min., the longest exposure)
o-Nitrobenzoic	Action slow; 7 min. required to form membranes in 50 per cent of eggs; almost no blastulæ formed up to 12 min.
m-Nitrobenzoic	11–12 min. (35–50 per cent)
p-Nitrobenzoic	9–10 min. (30–35 per cent)

With benzoic acid eggs were transferred to sea water at 2 minute intervals, from 2 to 30 minutes; with the other acids at 1 minute intervals, from 1 to 12 minutes.

of chlor-substitution. The relative ineffectiveness of *o*-nitro acid is difficult to understand, but is probably connected with its relatively great strength as an acid ($K = 6.3 \times 10^{-3}$). It is stronger than salicylic acid ($K = 1.06 \times 10^{-3}$) and formic acid ($K = 0.22 \times 10^{-3}$) and decidedly stronger than lactic acid ($K = 0.14 \times 10^{-3}$) which also is

ineffective as compared with the fatty acids. Of the first five fatty acids formic acid is the least effective in relation to the C_H of its solutions,[4] a peculiarity probably related to its greater strength. The comparative ineffectiveness of the mineral acids is well known; in this case the difficulty is one of penetration. The indications from membrane formation are that ortho-nitrobenzoic and lactic acids also penetrate the egg slowly. The factors determining the rate of penetration are insufficiently known at present. Surface activity is undoubtedly important, but the relation to ionization as such is uncertain, as indicated (*e.g.*) by the differences between salicylic acid and the equally strong ortho-benzoic acids.

Relation between Strength and Activating Effectiveness of Penetrating Acids.

If we again[4] compare the concentrations of pure acid solutions having equal rates of action, taking as the standard rate that corresponding to an optimum exposure in 10 minutes at 20°, the values (approximate) given in Table IX are obtained. This table includes the results of the earlier experiments with fatty acids and carbonic acid[1] as well as those of the present series. The second column gives the concentrations of the physiologically equivalent solutions, and the last column the calculated H ion concentrations of these solutions. While some degree of irregularity is shown, the C_H values are evidently closely similar; with the exception of formic acid and the two ortho-benzoic acids, all lie between 1.1 and 2.1 $\times 10^{-4}$ N and the majority between 1.6 and 2.1 $\times 10^{-4}$ N (pH = 3.7–3.8). The relation between the strength of the activating acids and their rate of action is thus a direct one, in conformity with the general idea that the rate of the activation reaction is determined by the intracellular C_H. We may infer that activation begins when a certain critical C_H is attained at the localized site of the reaction (apparently in the egg cortex) and proceeds at a rate which is closely proportional to this C_H. The reaction reaches its completion in a time determined by C_H and temperature, and in some way transforms the egg from the resting to the automatically developing state.

[4] Lillie,[1] Table XII, p. 364.

These estimates of C_H are subject to correction, since they do not take account of the increased dissociation which the weak acid almost certainly undergoes in the presence of the neutral salt; the possibility should also be considered that there are other factors within the egg influencing dissociation, such as the electrically polarized state of the structural surfaces at or near which the activation reaction occurs. The precise degree of the neutral salt influence is as yet unknown; but the physiological effects indicate that in the case of acetic, benzoic,

TABLE IX.

Approximate Concentrations of Acids Causing Complete Activation in 10 Minutes at 20°.

Acid	Concentrations	Dissociation constants	Calculated C_H of solutions
	M		($C_H \times 10^4$)
Formic	.0008	2.2×10^{-4}	4.2
Acetic	.0025	1.8×10^{-5}	2.1
Propionic	.0024	1.4×10^{-5}	1.8
Butyric	.0022	1.4×10^{-5}	1.75
Valeric	.0018	1.4×10^{-5}	1.6
Caproic	.0014	1.45×10^{-5}	1.4
Carbonic	.035	3.2×10^{-7}	1.1
Benzoic	.0005	6×10^{-5}	1.7
Salicylic	.00025	1.06×10^{-3}	2.1
o-Chlorobenzoic	.00045	1.32×10^{-3}	3.5
m-Chlorobenzoic	.0002	1.55×10^{-4}	1.15
p-Chlorobenzoic	.00025	0.93×10^{-4}	1.12
o-Nitrobenzoic	(?)	6.3×10^{-3}	(?)
m-Nitrobenzoic	.00035	3.5×10^{-4}	2.1
p-Nitrobenzoic	.00025	4.0×10^{-4}	1.7

and salicylic acids dissolved in 0.5 M NaCl it is equivalent to a two-fold or threefold increase in the dissociation constant.[5] This is clearly seen in the manner in which the action of the acid varies in the presence of varying concentrations of its salt (*cf.* next section).

[5] As just indicated, it is possible that the salt influence in the interior of the egg may be supplemented by other factors peculiar to the egg system. For example, the orientation of the adsorbed molecules of acid at the structural interfaces, with the carboxyl groups directed toward the water phase, as Harkins' theory requires, may increase dissociation, very much as various terminal substitutions are known to do. Combined with this influence would be that of the electrical polarization field at the phase boundary.

Action of Acids in Presence of Their Salts.

It was shown in the previous paper[1] that when Na acetate (.002 to .016 M) is added to activating solutions of acetic acid (.002 to .004 M) the rate of activation is increased to a moderate degree (10 to 20 per cent), in correspondence with the estimated proportional increase in undissociated acetic acid. Experiments with strong acid (HCl)and Na acetate (added separately to the isotonic NaCl-$CaCl_2$ solution) showed that the simple addition of H ions or acetate ions is ineffective, relatively or absolutely. Apparently activation is a consequence of the penetration of the undissociated molecules of acid followed by dissociation within the egg. The fact that the addition of acetate, which decreases the ionization of the acid in the external solution, does not retard but on the contrary accelerates the action of the acid within the egg is in itself an indication of the non-penetration (or very slow penetration) of the acetate ions.[6]

Experiments performed last summer with solutions of benzoic acid *plus* Na benzoate and of salicylic acid *plus* Na salicylate bear out this interpretation. On account of the greater strength of these acids, the addition of the Na salt to their solutions causes a greater proportional increase of undissociated molecules than in the case of acetic acid. This increase is greater with salicylic acid than with benzoic acid; in correspondence with this difference, the accelerating effect of the addition of the Na salt was found much greater with the former acid (*cf.* Fig. 1). The observed acceleration shows in both cases a satisfactory correspondence with that anticipated on the assumption that only the undissociated molecules in the solution are parthenogenetically effective. The acceleration, however, was in both cases greater than that calculated on the basis of the accepted dissocia-

[6] Some years ago, in a study of the toxic action of acids on infusoria (*Paramecium* and *Euplotes*), Miss Collett found that after the addition of non-toxic quantities of the salts of various weak acids (fatty acid, benzoic and salicylic acids) to toxic solutions of the same acids the resulting mixture was more toxic than could be explained by the acidity alone; and she interpreted this result as indicating toxicity of the undissociated acid molecules. In the case of salicylic acid and (in part) benzoic acid toxicity was definitely increased by adding the salt. These results also are in harmony with the view that only the undissociated molecules penetrate the cell freely (Collett, M. E., *J. Exp. Zool.*, 1921, xxxiv, 67).

tion constants, a result probably attributable mainly to the increased dissociation of the acid in the presence of the neutral salt (= 0.52 M NaCl).

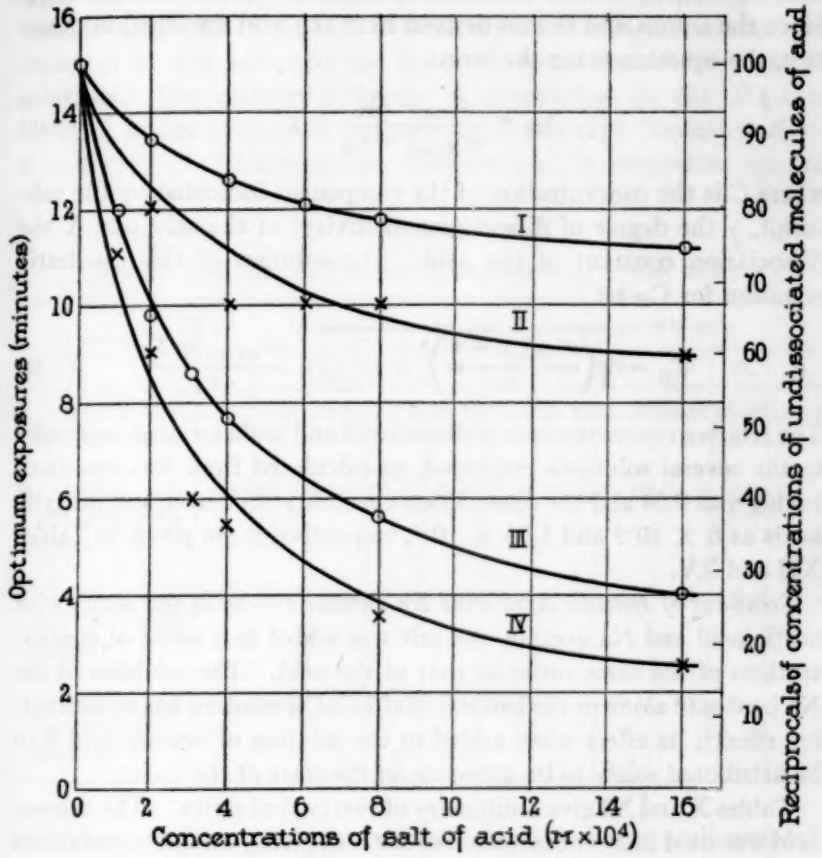

FIG. 1. Calculated and observed optimum exposures to .0004 M benzoic acid (Curves I and II) and .0002 M salicylic acid (Curves III and IV) in the presence of the Na salts of the acids. Ordinates, durations of exposures at 20°; abscissæ, concentrations of salt.

In estimating the dissociation of relatively strong acids like salicylic acid, and even of benzoic acid at the low concentrations employed in these experiments, it is necessary to take into account the anions

derived from the acid, as well as those from the added Na salt, in calculating the conditions at equilibrium.[7] The C_H is given by the ratio of the concentration of undissociated molecules of acid to that of the anions (from both sources), multiplied by the dissociation constant. Since the anions and H ions derived from the acid are equal in quantity, the equation takes the form:

$$C_H = \frac{C_{acid} - C_H}{\gamma C_{Na\ salt} + C_H} \times K \qquad (1)$$

where C is the concentration of the component indicated by the subscript, γ the degree of dissociation (activity) of the salt, and K the dissociation constant of the acid. The solution of this quadratic equation for C_H is:

$$C_H = \sqrt{\left(\frac{\gamma C_{Na\ salt} + K}{2}\right)^2 + KC_{acid}} - \frac{\gamma C_{Na\ salt} + K}{2} \qquad (2)$$

The relative concentrations of dissociated and undissociated molecules in the several solutions employed, as calculated from this equation, taking γ as 0.64 and the dissociation constants of benzoic and salicylic acids as 6×10^{-5} and 1.06×10^{-3}, respectively, are given in Tables XII and XV.

Solutions of Benzoic Acid Plus Na Benzoate.—As in the series with acetic acid and Na acetate, the salt was added in a series of concentrations of the same order as that of the acid. The addition of the Na benzoate alone to the isotonic NaCl-$CaCl_2$ solution has no activating effect[8]; its effect when added to the solution of benzoic acid is to be attributed solely to its influence on the state of the latter.

Tables X and XI give a summary of two typical series. The benzoic acid was used in a concentration of .0004 M, giving complete activation in 14 to 16 minutes in the pure solution. In the other solutions the concentrations of benzoate ranged from .0002 M to .0016 M. It will be observed that the addition of the salt increases the rate of activation very considerably (from 25 to 70 per cent) and that the increase is

[7] *Cf.* Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 2nd edition, 1922, pt. 1, 38. Furman, N. H., in Taylor, H. S., Treatise on physical chemistry, New York, 1924, ii, 833.

[8] The same is true of Na salicylate.

relatively greater than in the similar experiments with acetic acid; also that (as in the case of acetic acid) the degree of acceleration tends somewhat rapidly toward a limit as the salt content is increased. That the accelerating influence of the salt should be greater with benzoic than with acetic acid is to be expected from the greater dissociation of this acid and the lower concentration of its effective solutions. The calculated degree of dissociation in the .0004 M solution, in the absence of benzoate, is 30 per cent (assuming $K = 6 \times 10^{-5}$). If the dissociation were completely suppressed by the

TABLE X.

Benzoic Acid Plus Na Benzoate. Temperature 20° (June, 1926).

Composition of solutions (20°)	Durations of exposures (min.) and percentages of blastulæ										
	4	6	8	10	12	14	16	18	20	22	24
A. .0004 M benzoic acid (alone)	0	0	1–2	*Ca.* 5	20–30	*Ca.*50	70–80	70–80	50–60	15–20	*Ca.*10
B. .0004 M acid *plus* .0002 M Na benzoate	<1	20–30	40–45	65–70	60–70	30–40	25–30	*Ca.*10	*Ca.* 5	<1	0
C. .0004 M acid *plus* .0004 M Na benzoate	<1	10–15	50–60	50–60	*Ca.*50	25–35	15–20	<1	<1	0	0
D. .0004 M acid *plus* .0006 M Na benzoate	*Ca.*1	30–40	50–55	50–60	45–50	30–35	*Ca.* 1	0	0	0	0

addition of sufficient Na benzoate the concentration of undissociated molecules would become 100/70 of the original, an increase of *ca.* 43 per cent. This represents the limit to which increase of undissociated acid tends as the salt is added and explains why the rate of activation similarly tends toward a limit. Table XII gives the calculated dissociations of the added acid in the several solutions used (fourth column), together with the relative concentrations of undissociated acid, expressed as percentages of the free acid added (fifth column), and also (sixth column) in comparison with the undissociated

acid in the benzoate-free solution. The reciprocals ($\times 10^4$) of the latter values are also given (seventh column).

If we assume that the rates of activation (the reciprocals of the optimum exposures) in the several solutions are directly proportional to the concentrations of undissociated acid, the observed and the expected rates of activation may be compared. This is done in the two upper curves of Fig. 1. Curve II shows the variation in the durations of the optimum exposures with varying concentrations of benzoate; the points are the approximate optimum exposures in the

TABLE XI.

Benzoic Acid Plus Na Benzoate. Temperature 20° (June, 1926).

Composition of solutions (20°)	Durations of exposures (min.) and percentages of blastulæ										
	4	6	8	10	12	14	16	18	20	22	24
A. .0004 M benzoic acid (alone)	0	0	1–2	20–30	40–50	80–90	65–75	60–70	60–70	*Ca.*50	25–35
B. .0004 M acid *plus* .0004 M Na benzoate	<1	15–20	70–80	70–80	65–75	55–60	*Ca.*50	20–30	*Ca.* 5	0	0
C. .0004 M acid *plus* .0008 M Na benzoate	1–2	30–40	*Ca.*50	65–75	65–75	*Ca.*50	30–40	10–15	*Ca.* 5	0	0
D. .0004 M acid *plus* .0016 M Na benzoate	<1	30–40	55–60	60–70	*Ca.*50	20–30	5–10	0	0	0	0

different solutions (*cf.* Tables X, XI, and Column 2 of Table XII). Curve I represents the reciprocals of the calculated concentrations of undissociated molecules; the concentration in the benzoate-free solution is given the value 100 (scale on right of figure) and is made to correspond with the observed optimum exposure (15 minutes) in the benzoate-free solution. If the dissociation were correctly given by the calculation, the two curves should coincide. It is evident, however, that the actual acceleration is greater in every case than that calculated. For example, the addition of .0016 M Na benzoate shortens the

optimum exposure from 15 minutes to *ca.* 9 minutes, an increase in the rate of activation of *ca.* 70 per cent, while the calculated increase in undissociated molecules is only 35 per cent (*cf.* Column 6, Table XII). The fact that the rate of activation is greater than that estimated is, however, readily understood if we assume that the dissociation of the acid is increased in the presence of the relatively high concentration (*ca.* 0.5 M) of neutral salt.[9] If the constant of benzoic acid is regarded as 2×10^{-4}, instead of 6×10^{-5}, the observed and the calculated curves agree closely. A similar disparity between the

TABLE XII.

Activation by .0004 M Benzoic Acid in Presence of Na Benzoate.

Concentration of Na benzoate	Durations of optimum exposures (*ca.*) (T)	Relative rates of activation $\left(\frac{15}{T} \times 100\right)$	Acid dissociated γ	Acid undissociated $(100-\gamma)$	Relative concentrations undissociated acid $\frac{(100-\gamma)100}{70}$	Reciprocals of concentrations undissociated acid $\frac{70}{(100-\gamma)100} \times 10^4$
M	*min.*		*per cent*	*per cent*		
0	14–16 (av. = 15)	100	30	70	100	100
.0002	12	125	21.75	78.25	112	89+
.0004	10	150	15.75	84.25	120	83+
.0006	<10	150+	12.25	87.75	125	80
.0008	<10	150+	9.75	90.25	128	78
.0016	8–10 (av.9)	170	5.5	94.5	135	74

observed and the calculated rates of activation is seen in the experiments with salicylic acid (Curves III and IV, Fig. 1) and acetic acid.[5]

Solutions of Salicylic Acid Plus Na Salicylate.—Four series of experiments with .0002 M salicylic acid and Na salicylate ranging from .0001 M to .0016 M were performed during June, 1926, at a time when the eggs were in good and uniform condition. The results of these experiments agree closely; they all show a rapid progressive increase in the rate of activation with increasing addition of Na salicylate, the relative acceleration being decidedly greater than in the corresponding experiments with benzoic acid (Fig. 1) and tending more slowly toward

[9] *Cf. e.g.*, Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipzig. 6th edition, 1926, 55.

a limit. Tables XIII and XIV give records of two of these series. In the absence of salicylate .0002 M salicylic acid shows an optimum effect at exposures of 14 to 16 minutes. The addition of .0002 M to .0003 M salt approximately doubles the rate of activation; that of .0008 M to .0016 M increases it from four to six times. This relatively great acceleration, as contrasted with the moderate acceleration observed with acetic and benzoic acids, is in accordance with the much higher dissociation constant ($K = 1.06 \times 10^{-3}$) of salicylic acid. A reference to the calculated percentages of dissociation given in Table

TABLE XIII.

Salicylic Acid Plus Na Salicylate. Temperature 20° (June, 1926).

Composition of solutions (20°)	Durations of exposures (min.) and percentages of blastulæ											
	2	4	6	8	10	12	14	16	18	20	22	24
A. .0002 M salicylic acid (alone)	0	0	0	<1	*Ca.*10	*Ca.*20	35–45	60–70	50–60	40–50	20–25	10–15
B. .0002 M acid *plus* .0001 M Na salicylate	0	0	<1	15–20	35–40	45–50	*Ca.*10	5–10	*Ca.* 5	*Ca.* 1	*Ca.* 1	0
C. .0002 M acid *plus* .0002 M Na salicylate	0	<1	*Ca.*10	35–45	25–35	*Ca.*10	*Ca.*10	*Ca.*10	*Ca.* 5	0	0	0
D. .0002 M acid *plus* .0003 M Na salicylate	<1	*Ca.* 5	25–35	15–20	*Ca.*10	*Ca.*10	*Ca.* 5	*Ca.* 1	0	0	0	0

XV shows that the possible increase in undissociated molecules in the .0002 M solution is from 14 per cent (in the salt-free solution) to 100 per cent (with complete suppression of dissociation),—*i.e.*, about seven times. The calculated increase resulting from the addition of .0016 M salicylate is 52/14,—nearly four times (Column 5, Table XV). The actual increase in rate of activation is, however, greater than this,—from five to six times, indicating again an increased dissociation of the acid in the neutral salt solution. A similar disparity between the observed and the calculated rates of activation is seen in every

solution of the series. Apart from this discrepancy, which can be remedied by assigning a higher value (*ca.* 2×10^{-3}) to the dissociation constant, the agreement between the observed and calculated rates of activation is satisfactory. Curve IV in Fig. 1 shows the variation in

TABLE XIV.

Salicylic Acid Plus Na Salicylate. Temperature 20° (June, 1926).

Composition of solutions (20°)	Durations of exposures (min.) and percentages of blastulæ											
	2	4	6	8	10	12	14	16	18	20	22	24
A. .0002 M salicylic acid (alone)	0	0	*Ca.* 1	15–20	30–35	*Ca.*50	50–60	25–30	15–20	2–4	<1	0
B. .0002 acid *plus* .0008 M Na salicylate	15–20	15–20	<5	0	0	0	0	0	0	0	0	0
C. .0002 M acid *plus* .0016 M Na salicylate	25–30	<5	0	0	0	0	0	0	0	0	0	0

TABLE XV.

Activation by .0002 M Salicylic Acid in Presence of Na Salicylate.

Concentration of Na salicylate	Durations of optimum exposures (*ca.*) (T)	Relative rates of activation $\left(\frac{15}{T} \times 100\right)$	Acid dissociated (γ)	Acid undissociated ($100-\gamma$)	Relative concentrations undissociated acid $\frac{(100-\gamma)100}{14}$	Reciprocals of concentrations undissociated acid $\frac{14}{(100-\gamma)100} \times 10^4$
M	*min.*		*per cent*	*per cent*		
0	14–16 (av.15)	100	86	14	100	100
.0001	10–12	136	82.5	17.5	125	80
.0002	8–10	167	78	22	157	64
.0003	*Ca.* 6	250	75.5	24.5	175	57
.0004	5–6	272	72.5	27.5	196	51
.0008	3–4	428	62.5	37.5	268	37+
.0016	2–3	600	48	52	372	27

the observed optimum exposures in the different mixtures; in Curve III the calculated exposures are plotted, assuming that they are proportional to the reciprocals of the concentrations of undissociated acid (Column 7 of Table XV).

General Relations of External Acidity to Physiological Activity.

The conditions under which the starfish egg is activated by acids are probably not peculiar to this cell, and in fact the foregoing observations show certain significant parallels with the conditions found in other living systems which respond definitely to variations of external acidity. For example, in the vertebrate respiratory center increase in the rate of physiological activity is also known definitely to follow increase in the external concentration of penetrating acids. Under the usual conditions, increase in the external C_H accelerates the respiratory rhythm; yet we often find experimentally a similar increase of activity even when the change of external C_H is absent or in the opposite direction.[10] Such facts have recently led to a modified conception of the relation of acidity to the respiratory rhythm. Although this rhythm is still regarded as controlled by variations of H ion concentration, it seems necessary to conclude that the essential factor to be considered is not the C_H of the external medium, but that existing within the cells of the center themselves. This, however, is only indirectly dependent on the external C_H. Jacobs[11] has recently furnished clear demonstration that the C_H of the cell interior may vary widely from that of the surrounding medium, especially when the latter contains the salts of weak acids or bases. What is true of the various cells where this demonstration can be made is in all likelihood also true of the nerve cells of the respiratory center. We may assume that these cells, possibly deficient in buffering compounds, are specially sensitive to variations of internal C_H, and vary their rate of activity correspondingly.

In the starfish egg we find conditions which in many respects are closely analogous with those just considered. In pure solutions of the activating acid increase in acidity is followed by a closely proportional increase in the rate of activation. From this fact, considered alone, we might conclude that the rate of activation is directly determined by the external C_H. But in the presence of the salts of the acid, although

[10] For a recent discussion *cf.* Gesell, R., *Physiol. Rev.*, 1925, v, 551; *cf.* p. 552 *et seq.* The effect just named is well illustrated in Loevenhart's work on the stimulation of the respiratory center by cyanide; *cf.* Gasser, H. S., and Loevenhart, A. S., *J. Pharmacol. and Exp. Therap.*, 1914, v, 239.

[11] Jacobs, M. H., *Am. J. Physiol.*, 1920, li, 321; liii, 457.

the relation of the rate of activation to the total concentration of added free acid is the same as before, we find a complete independence of external C_H.[1] In fact, solutions containing a fixed concentration of free acid and varying quantities of Na salt show a precisely contrary relation; increase in the rate of activation is then correlated not with increase but with decrease of external acidity. The two cases, however, are easily reconciled if we recognize that activation is dependent on the penetration not of the ions but of the undissociated molecules of the acid. These furnish the activating ions by their dissociation within the egg.

Apparently only the undissociated molecules of acid penetrate freely into the egg; there is increasing evidence that this condition is widespread among living cells.[12] In cells having this type of permeability variations of intracellular acidity, with corresponding variations in physiological activity, would tend to follow variations in the concentration of the undissociated molecules of acid or base in the external medium. This consideration explains why in the starfish egg a direct correlation between external C_H and rate of activation is found only under those conditions in which the concentrations of undissociated molecules and of H ions are also directly correlated, as in the pure solutions of the acid. In solutions containing the salt of the acid the correlation between C_H and undissociated acid may be an inverse one, as in the cases considered above: we then observe the rate of activation to follow the concentration of the undissociated molecules. External acidity is thus, taken by itself, an uncertain and variable index of intracellular acidity; it is, however, the latter which controls the rate of the physiological process.

Such a view implies that the production of acid within the cell would have the same physiological effect as the penetration of acid molecules from without. Apparently this is what occurs in the heat activation of the starfish egg. In the case of the respiratory center, Gesell has recently upheld strongly on both theoretical and experimental grounds the view that the intracellular production of lactic

[12] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1925–27, viii, 131. Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, ix, 255. Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 234. Irwin, M., *J. Gen. Physiol.*, 1926, ix, 561. Smith, H. W., *Am. J. Physiol.*, 1925, lxxii, 347; 1926, lxxvi, 411.

acid is the essential factor in the increase of respiration accompanying asphyxia.[10,13] It is clear that in considering the problem of the relation of H ions to cellular activity we must take into account not only the permeability of the cell to acids, and other features such as the buffering capacity of its protoplasm, but also the nature and quantity of the acids produced in its special type of metabolism under varying conditions.[14]

SUMMARY.

1. Comparison of the rates of activation of unfertilized starfish eggs in pure solutions of a variety of parthenogenetically effective organic acids (fatty acids, carbonic acid, benzoic and salicylic acids, chloro- and nitrobenzoic acids) shows that solutions which activate the eggs at the same rate, although widely different in molecular concentration, tend to be closely similar in C_H. The dissociation constants of these acids range from 3.2×10^{-7} to 1.32×10^{-3}.

2. In the case of each of the fourteen acids showing parthenogenetic action the rate of activation (within the favorable range of concentration) proved nearly proportional to the concentration of acid. The estimated C_H of solutions exhibiting an optimum action with exposures of 10 minutes (at 20°) lay typically between 1.1×10^{-4} M and 2.1×10^{-4} M (pH = 3.7–3.96), and in most cases between 1.6×10^{-4} M and 2.1×10^{-4} M (pH = 3.7–3.8). Formic acid ($C_H = 4.2 \times 10^{-4}$ M) and *o*-chlorobenzoic acid ($C_H = 3.5 \times 10^{-4}$ M) are exceptions; *o*-nitrobenzoic acid is ineffective, apparently because of slow penetration.

3. Activation is not dependent on the penetration of H ions into the egg from without, as is shown by the effects following the addition of

[13] Gesell, R., *Science*, 1926, lxiii, 58; *Am. J. Physiol.*, 1923, lxvi, 5. McGinty, D. A., and Gesell, R., *Am. J. Physiol.*, 1925–26, lxxv, 70.

[14] Warburg and his associates have recently shown that various types of carcinoma cell are characterized by an unusually high production of lactic acid, under both anaerobic and aerobic conditions (Warburg, O., Posener, K., and Negelein, E., *Biochem. Z.*, 1924, clii, 309). They infer that the transformation of the normal resting tissue cell into the rapidly proliferating cancer cell is dependent on a change from the normal metabolism to an acid-producing or glycolytic type. The accumulation of acid within the cell would thus be the condition activating cell division in tumor-producing tissue cells as well as in the starfish egg.

its Na salt to the solution of the activating acid (acetic, benzoic, salicylic). The rate of activation is *increased* by such addition, to a degree indicating that the parthenogenetically effective component of the external solution is the undissociated free acid. Apparently the undissociated molecules alone penetrate the egg freely. It is assumed that, having penetrated, they dissociate in the interior of the egg, furnishing there the H ions which effect activation.

4. Attention is drawn to certain parallels between the physiological conditions controlling activation in the starfish egg and in the vertebrate respiratory center.

A RELATIONSHIP BETWEEN CIRCUMFERENCE AND WEIGHT IN TREES AND ITS BEARING ON BRANCHING ANGLES.

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When a tree, at a point where the circumference is c_0, divides into two branches (c_1 and c_2), what relationship exists between c_0 and $c_1 + c_2$? In order to answer this question, which has a definite bearing on problems of tree form, it is convenient to investigate first the relationship between the circumference at some point and the weight, w, of all the parts of the tree peripheral to this point.

Accordingly measurements were made, 116 in all, on nine kinds of trees; namely, aspen, bitternut, hickory, oak, ash, maple, cedar, hornbeam, and beech. The largest tree measured had a circumference of 56.4 cm. where cut, and the whole tree weighed 120 kg. The smallest measurements were made on the stems of leaves,—for example, circumference of stem = 0.25 cm., weight of leaf = 0.18 gm. All the data thus obtained are included in Fig. 1.

Our procedure was of the simplest character. Whole trees of varying size, or branches, or leaves, were taken entirely at random from the vicinity. The only criterion of selection was that the specimen should not appear to have been recently injured. The circumference was measured by a tape, encircling the bark, at the point of section; or, for small specimens, the diameter was measured by calipers and the circumference subsequently calculated. The specimen was then weighed on one of three balances according to size. The season was midsummer, 1926; the place, Grindstone Island, N. Y.

Plotting logarithms; *i.e.*, log (weight in gm.) *vs.* log (circumference in cm.), the points fall close to a straight line. A statistical treatment yields the following numerical characteristics:

Mean value of log c in the observations	= 0.161
" " " log w " " "	= 1.250

Standard deviation of log c	= 0.507
" " " log w	= 1.263
Mean product of simultaneous deviations	= 0.637
Correlation coefficient	= 0.99

From these figures one obtains, for the best linear relation between log w and log c, the equation:

$$\log w = 2.49 \log c + 0.850; \text{ or, } \quad w = 7.08\, c^{2.49} \qquad (1)$$

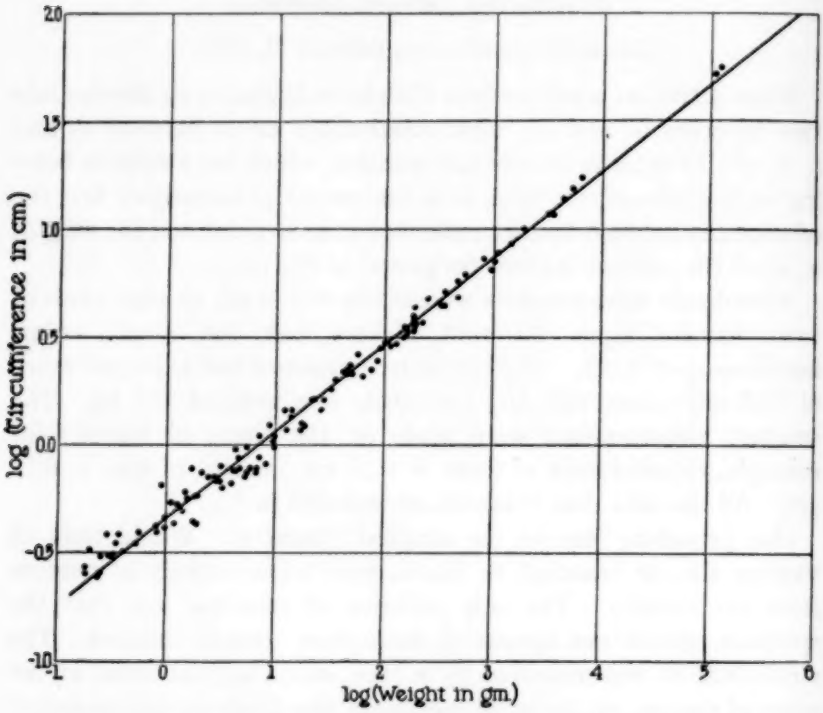

FIG. 1. The line is drawn according to equation (2). The trend toward a cube law relation among the observations appearing in the lower left portion of the chart may be significant.

The probable error of log w is $\pm$ 0.08,—*i.e.*, if from the measured circumferences estimates of the weight are calculated by equation (1), then half of the actual observed weights fall within the limits +20 per cent and −17 per cent of the calculated values. It will be seen from Fig. 1 that the error is greater for the small pieces, and less

for the large pieces. We observed also that deviations from equation (1) occurring in various parts of the *same* individual tree are sufficiently large to mask any systematic differences between the different *kinds* of trees that were studied. Furthermore, the equation holds as well for stems bearing nuts as for stems bearing leaves.

Returning to the opening question, the solution, inherent in equation (1), is given by the relation:

$$c_0^{2.49} = c_1^{2.49} + c_2^{2.49} \qquad (2)^*$$

This follows from the fact that, if a main stem or trunk is cut near a point of branching and weighed (w_0), and then if the branches are weighed separately (w_1 and w_2), w_0 must equal $w_1 + w_2$.

Equation (2) describes, for the class of trees studied, one special characteristic of branching. The exponent 2.49, being greater than 2, indicates, for example, that the total cross-sectional area of the branches becomes progressively greater at each branching. To express this property we may say that trees follow statistically a "2.5 power law of branching."

Another characteristic of branching is the equation which describes the angles of branching. In a previous paper[1] this problem was discussed in reference to the *arterial* system in animals, and the following equations, of which two only are independent, were deduced:

$$\cos x = \frac{c_0^4 + c_1^4 - c_2^4}{2\,c_0^2\,c_1^2};\ \cos y = \frac{c_0^4 - c_1^4 + c_2^4}{2\,c_0^2\,c_1^2};\ \cos(x+y) = \frac{c_0^4 - c_1^4 - c_2^4}{2\,c_1^2\,c_2^2} \qquad (3)$$

where c_0, c_1, and c_2 are the circumferences of the main stem and the two branches into which it divides; and where x and y are the angles made by the branches (c_1 and c_2) with the line of direction of the stem. The angle $(x + y)$ is, of course, the angle included between the two branches. These equations, for our present purpose, may be considered as being deduced from the assumption that the branching system connecting three points shall, for given circumferences of the stem and branches, require the least volume of wood.

*Once obtained, this relation may be roughly checked by simple measurement, without weighing or cutting, on large trees.

[1] The physiological principle of minimum work applied to the angle of branching of arteries, Murray, C. D., *J. Gen. Physiol.*, 1925–26, ix, 835.

If now equations (2) and (3) are combined one can, at the expense of loss of generality, solve directly for the angle as a function of some convenient ratio such as c_1/c_0 or c_1/c_2. The steps are shown in the previous paper. But in that paper, instead of a 2.5 power law (equation (2)), a cube law (theoretically deduced for the arterial system) was used. In either case certain qualitative rules hold which describe in words the variations in the angles accompanying variations in the ratio c_1/c_2, etc. An interesting and convenient illustration of these

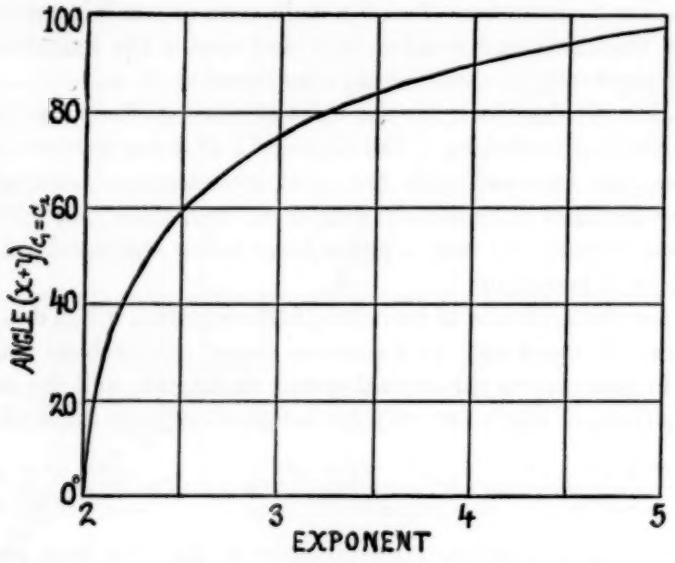

FIG. 2. The curve shows the relation between the *angle* $(x + y)$, given that $c_1 = c_2$, calculated by equations (2) and (3), and the *exponent*, considered variable, occurring in equation (2).

rules may be seen in the branching of the veins of leaves. There remains only to be observed the fact that, in changing from a cube law to a 2.5 power law, the calculated angles, for any given ratio of circumferences, become smaller,—a fact corresponding to a difference between the branching of arteries and of trees. For example, solving equation (3) for the angle $(x + y)$ when $c_1/c_2 = 1$, we find for a cube law angle $(x + y) = 75°$, for a 2.5 power law angle $(x + y) = 59°$. The curve in Fig. 2 shows this relation.

SUMMARY.

Observation reveals a linear relationship between the logarithm of the circumference of a tree, branch, or leaf stem, and the logarithm of the weight of the tree, branch, or leaf. The bearing of this on the angles of branching in trees is discussed.

MICRURGICAL STUDIES IN CELL PHYSIOLOGY.

III. The Action of CO_2 and Some Salts of Na, Ca, and K on the Protoplasm of Amœba dubia.*

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(Accepted for publication, January 28, 1927.)

The first paper (1) of this series described the action of the chlorides of Na, K, Ca, and Mg on the protoplasm of *Amœba dubia* as determined by micrurgical technique. A study of the effect of other salts of some of these cations forms the basis of this report.[1] Among those tested were a few which are often employed in preparing buffer solutions and some of general physiological interest, particularly solutions containing phosphate, borate, lactate, acetate, bicarbonate, carbonate, and CO_2. The details of the apparatus, its manipulation, and the terminology used have been fully described in a previous publication (1) to which the reader is referred.

Immersion Experiments.—When amebæ are immersed in toxic concentrations of any of the Na salts used in these experiments, the same effect is obtained as that found with NaCl (1), *viz.*, rounding, quiescence, and sinking of the heavier granules. In general, the toxicity of the phosphates depends upon the relative amount of Na in the salt (Table I). The only exception to this is the marked toxicity of NaH_2PO_4 in concentrated solutions due to the acidity of this salt (1). Alkalinity alone, as has been shown previously (1), is not a factor in the production of toxic effects. The phosphate ion may be a factor in contributing to the toxicity of these salts, since even in

*The ameba that has been used in the studies of this series previously identified as *Amœba proteus* is the form described by Schaeffer in his book on Amœboid movement (1920) as *Amœba dubia*.

[1] We wish to thank Mr. Kenneth Blanchard for his help in preparing and analyzing some of the chemicals used in this work.

the more dilute solutions amebæ do not appear as healthy as in control solutions containing an equivalent amount of Na.

The borate is more toxic than any other salt used in this series (Table I), a condition which is in keeping with the long established use of borates as an antiseptic.

$NaHCO_3$ and Na_2CO_3 are very toxic within the first 24 hours of immersion (Table I). Even in dilute solutions, ranging from M/1024 to M/8192, the amebæ become round and sluggish. In a few hours, however, those which have retained intact plasmalemmæ recover and remain living and well. Fresh amebæ placed in these solutions which have stood for several hours are not affected. This fact indicates

TABLE I.

Viability of Amebæ Immersed in Decreasing Concentrations of Salts.

Salts	Dead in						Living through 5 days
	1 hr.	1 day	2 days	3 days	4 days	5 days	
Monosodium phosphate	M/28	M/512	—	—	—	—	M/2048
Disodium phosphate	M/24	M/1024	—	—	—	—	M/2048
Trisodium phosphate	M/384	M/1536	—	—	—	—	M/2048
Sodium borate	M/768	M/1536	—	—	—	—	M/3072
Sodium bicarbonate	M/256	M/512	—	—	—	—	M/1024
Sodium carbonate	M/384	—	—	—	—	—	M/1024
Sodium lactate	M/4	M/32	—	—	M/384	—	M/1024
Sodium acetate	M/4	M/8	M/32	M/128	M/256	—	M/512
Calcium acetate	M/2	M/16	—	—	—	—	M/48
Calcium lactate	—	M/7.5	—	M/15	—	—	M/30

that a change takes place in solutions of Na_2CO_3 and $NaHCO_3$ on standing and the implication is that the loss of CO_2 is a factor in the change. To determine this, amebæ were immersed in water saturated with CO_2. Such a solution, tested immediately after preparation, had a pH of 4.8. The amebæ showed the same effects as in the carbonates. With the gradual increase in alkalinity due to the loss of CO_2, those amebæ recover whose plasmalemmæ have remained intact.

The lactate of Na, although not markedly toxic (Table I), can inhibit activity even in very dilute solutions.

The acetate is the least toxic of all the Na salts used (Table I).

Ca acetate and Ca lactate resemble $CaCl_2$ (1) in their non-toxicity

(Table I). It is interesting to note, however, that the lactate is the only salt of Ca which has a destructive action on the plasmalemma.

Injection Experiments.—In general the injection of the Na salts produces the same effect as that of the chloride (1). With NaH_2PO_4, however, this is neither marked nor sustained. The injection of this salt is characterized by an elevation of the plasmalemma with the appearance of a subjacent hyaline zone and the formation of a distinct membrane-like film or boundary around the granuloplasm within the hyaline zone. This boundary breaks down during recovery when flowing movements of the granuloplasm fill the hyaline zone with granules, Fig. 1. Na_2HPO_4 and Na_3PO_4 are more toxic than NaH_2PO_4 (Table II), as is to be expected because of their increased Na content. These salts do not form the granuloplasmic boundary peculiar to NaH_2PO_4.

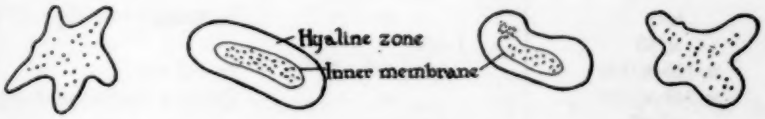

FIG. 1. The production of an inner membrane upon injection of NaH_2PO_4 into an ameba.

The borate is much more toxic than the phosphates to the interior of the ameba (Table II).

$NaHCO_3$ and Na_2CO_3 are more toxic than the other Na salts except borate because of their marked solvent action on the plasmalemma (Table II). To determine the rôle of the carbonate alone in producing this effect, bubbles of CO_2 gas were introduced into the ameba. In the cytoplasm they shrink, apparently by going into solution. If the injected bubble is larger in size than that of the nucleus, the plasmalemma fades and disappears over the entire ameba and the granuloplasm scatters. When a very small bubble of CO_2 is injected into the middle region, the plasmalemma disappears only at one end, usually the hind end of the ameba and the ameba recovers. The shrinking bubble tends to disappear before the surface breaks, Fig. 2. Control injections of bubbles of air do not affect the ameba unless the bubble is large enough to burst the ameba.

TABLE II.

Recovery of Amebæ from Injection of Decreasing Concentrations of Salts.

Salts	Recovery from			Water effect
	Small injection	Moderate injection	Large injection	
Monosodium phosphate	M/2	M/4	M/12	M/1024
Disodium phosphate	M/4	M/16	M/32	M/1280
Trisodium phosphate	M/4	M/16	M/64	M/768
Sodium borate	M/16	M/32	M/64	M/512
Sodium bicarbonate	M/16	M/32	M/64	M/256
Sodium carbonate	M/16	M/32	M/64	M/256
Sodium lactate	—	M	M/4	M/2048
Sodium acetate	M	M/3	M/8	M/128

Concentration	Pinching off effect	
	Calcium lactate	Calcium acetate
M/1	—	In 20 sec.
M/60	1 min.	—
M/120–M/130	Attempted only	In 1–2 min.
M/240–M/260	—	Delayed or attempted only
M/480	No attempt	—
M/520	—	No attempt

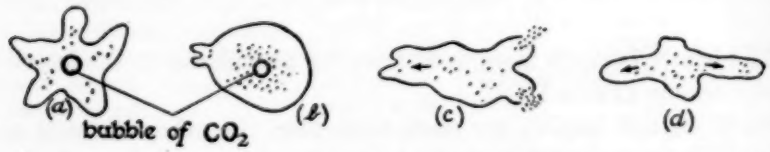

FIG. 2. The effect of injecting a small bubble of CO_2 into an ameba: (*a*) immediately after injection; (*b*) beginning rounding of ameba and sinking of granules, shrinking of bubble; (*c*) disappearance of bubble, beginning flow of ameba, breaks in plasmalemma; (*d*) recovery.

FIG. 3. The antagonistic effect of lactate on the pinching off reaction of Ca: (*a*) before injection; (*b*) beginning pinching off of solidified area; (*c*) inflow into injected area from healthy portion pushing back sluggish, constricting membrane of the stalk; (*d*) beginning incorporation; (*e*) recovery.

The lactate of Na, although relatively non-toxic (Table II), has a distinct quieting effect on the movement of the ameba and a solvent action on the plasmalemma.

The acetate is the least toxic of all the Na salts tested in this series.

Both Ca acetate and lactate have the same action as $CaCl_2$ (1), *viz.*, a solidification of the injected area which is pinched off by the living remnant. The rate of pinching off with the lactate, however, is slower than that with the acetate. This delay seems to be related to the visible sluggishness of the plasmalemma produced by the lactate. The pinching off process is frequently interrupted by an inrush of internal protoplasm. This frequently results in a failure to pinch off and, ultimately, in a consequent incorporation of the solidified material even with relatively concentrated solutions of Ca, Fig. 3.

In general, the effects of injecting the K salts were the same as those obtained with the Na salts except for the greater stability exhibited by the plasmalemma (1).

DISCUSSION.

It is evident that the predominant action of the salts is that of the cation. The anion may modify this effect without apparently changing its fundamental nature. Many of the salts tested are generally used as buffers. The usual strength of a buffer solution (M/20) is non-toxic when injected into the ameba. In immersion work, however, the buffer salts are too toxic to be used except in very dilute concentrations.

Some of the salts show individual peculiarities which are of interest. For equivalent concentrations of Na, the phosphates are more toxic in immersion experiments than the chloride. The probability that phosphates penetrate very slightly (2) suggests that their toxicity may be due to the extraction of substances, for example, Ca, from the ameba. A remarkable effect is the production of a membrane-like structure around the granuloplasm within the ameba when the acid phosphate is injected. This is perhaps due to a gelation or precipitation of some substance on the surface of the granuloplasm by virtue of an interaction with the injected phosphate. Whether the phosphate reacts with the Ca in the protoplasm it is not certain but in connection with this occurrence it is interesting to recall that an acid medium tends to accentuate the typical solidifying action of Ca (1).

The action of CO_2 and the carbonates in dissolving the plasmalemma is most significant because of the fact that CO_2 is more soluble in organic solvents than in aqueous solutions (3). This is evidence for the lipoid nature of the plasmalemma. Lillie (4) has shown in his work on the activation of starfish eggs by acids that CO_2 behaves like a fatty acid. However, the marked toxicity of CO_2 (5–7), has usually been attributed to its great penetrating power, thus implying that CO_2 exerts its toxic action on the interior. In fact, Jacobs (8) shows that CO_2 can enter and leave the cell with complete reversibility. This varies considerably with the cell used (9). The injection experiments indicate that in the ameba penetration into the interior is not the important factor in the production of lethal effects. In the ameba the ability to revert to normal depends upon the maintenance of an intact plasmalemma. The ameba is irreversibly injured only when CO_2 destroys the plasma membrane. This emphasizes again the importance of the surface in the maintenance of the life of the cell (10).

The lactates also act on the surface of the cell. Amebæ, immersed in these salts, are characterized by their sluggish plasmalemmæ. This is of interest in connection with Lillie's finding that lactic acid is relatively ineffective in activating the starfish egg, a condition which he attributes to difficulty of penetration. The dispersive action of the lactate on the plasmalemma is most evident when this salt is brought by injection into contact with the inside of the plasmalemma. This dispersing effect is further seen when amebæ are immersed in Ca lactate. This is the only Ca salt in which dead amebæ show a disrupted plasmalemma.

The relative non-toxicity of sodium acetate is rather surprising when one considers the frequent reports of its marked activity (4, 11). Loeb (12), however, pointed out that sodium acetate acts exactly like the chloride in its depressing effect on the viscosity of gelatin if the pH is kept constant. Furthermore, it is difficult to compare results from different materials or even different functions of the same material. Cohen and Clark, for example, point out that the effect of pH upon specific fermentative processes, upon reproduction in its several stages, and upon death, must be kept distinct. This may well be kept in mind in dealing with any factor.

CONCLUSIONS.

I. *Plasmalemma.*

1. Of the salts used in these experiments the anions have only a modifying effect on the cations. The dispersive action of Na and, to a lesser extent, of K, predominates. Borate increases the toxicity of Na and acetate decreases it.

2. CO_2 and carbonates dissolve the plasmalemma readily.

3. Na lactate tends to dissolve the surface especially when brought into contact with it from the interior by injection.

Lactate antagonizes the stimulating effect of Ca on the plasmalemma.

II. *The Internal Protoplasm.*

4. Acid phosphate of Na and K, when injected, causes a membrane to form around the granular endoplasm within the ameba.

5. Na borate increases the toxicity of Na inside the cell.

6. Bubbles of CO_2, injected into the cell, cause an increase of fluidity of the internal protoplasm. These bubbles shrink and disappear from the cell more readily than air bubbles.

7. The anions modify the typical cation effect. Carbonates accentuate the liquefying and solvent action of Na.

Phosphates prevent a complete rounding of the ameba caused by Na.

Lactate inhibits the solidification and pinching off effect caused by Ca.

III. *Physiological Significance of Salts.*

8. The buffer salts can be injected in high concentrations without toxic effects but amebæ can be immersed in them only in very dilute solutions without injury.

9. The inhibiting action of lactate and the dispersive effect of CO_2, carbonates, and lactate on the plasma membrane, must be of importance in a consideration of the functions of the organism and perhaps in the production of pathological changes.

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MICRURGICAL STUDIES IN CELL PHYSIOLOGY.

IV. Colorimetric Determination of the Nuclear and Cytoplasmic pH in the Starfish Egg.*

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An essential feature in determining the hydrogen ion concentration of protoplasm is the maintenance of a normal condition of the protoplasm during the procedure. Results obtained by immersing cells in solutions of dyes have been inadequate owing to the lack of satisfactory indicators to which living cells are freely permeable (1). Attempts have been made to overcome this difficulty by artificially altering the permeability of living cells (2). Any procedure, however, which exposes the cells to abnormal conditions may seriously affect the results obtained. The existence of natural dyes in the tissues has been utilized (3, 4) but has not given definite results. Some investigators have made both potentiometric and colorimetric determinations of cellular extracts (5, 6).

Recently, Vlès and his coworkers (7–10) have introduced a method (méthode microscopique d'écrasement) by means of which echinoderm eggs, immersed in an indicator solution, are carefully crushed between the cover slip and slide of a compressorium. As soon as the egg bursts pressure is released whereupon the dye passes in through the breaks over the surface of the egg. The objection that the pH of crushed cells may be quite different from that of the living protoplasm has already been considered by the Needhams (11). The results obtained by the micrurgical technique have brought out the importance of the plasma membrane for the maintenance of protoplasm (12–14). If a cell is crushed so that the plasma membrane disintegrates, the

* From the Eli Lilly Research Division, Marine Biological Laboratory, Woods Hole.

exuding material either merges with the environing medium and is destroyed or forms spherules of normal appearing protoplasm walled off by new surface membranes. From experiments described in this paper these spherules are no more permeable than the protoplasm in its original condition. The disintegrated material scatters or rounds up into droplets which swell and burst.

A potentiometric determination of the pH with the aid of the micrurgical apparatus has been made possible recently by the development of the micro electrodes of Ettisch and Péterfi (15) and Taylor (16). However, the use of micro electrodes thus far has not met with success in the determination of the pH of the protoplasm.

The micro injection of indicator dyes into the protoplasm of cells has met with considerable success. Kite (17) was probably the first to inject dyes into the protoplasm of a cell. With a meagre supply of indicators at his disposal he concluded that the interior of the ameba is faintly alkaline. More recently Schmidtmann (18, 19) introduced solid particles of dyes into mammalian tissue cells. He obtained values of pH varying from 5.9 to 7.8 in different cells. The Needhams used aqueous solutions of the Clark and Lubs series of indicator dyes and determined the internal pH of a number of marine ova to be 6.6 ± 0.1.

The investigations described in this paper were carried out principally because of the discrepancy between the results obtained by the Needhams and those of other investigators—notably Vlès, Reiss, and Vellinger (7–10)—and because of the desirability of also determining the pH of the nucleus.

A. Methods and Material.

The experiments were performed on the eggs of the echinoderm *Asterias forbesii*. The eggs to be injected with indicator solutions were immersed in hanging drops of normal or of acidified sea water. The pH of normal sea water when determined colorimetrically is 8.4 and, potentiometrically, 8.2 (20). To obtain acid sea water, KH_2PO_4 was added.

The dyes used were neutral red and those of the Clark and Lubs series of pH indicators covering the range from 4.4 to 8.4 (Sörensen units), *viz.*, methyl red, brom cresol purple,[1] brom thymol blue, phenol red, and cresol red. The neutral

[1] A peculiar feature of brom cresol purple is that the color of its alkaline range under the microscope appears distinctly blue rather than purple.

red was made up in a saturated aqueous solution and NaOH was added until the solution changed from a red color to a deep orange red with no sign of a precipitate. All the Clark and Lubs' indicators used were obtained from Hynson, Westcott, and Dunning, Baltimore. The dyes were prepared according to Clark (20) in 0.4 per cent aqueous solution with a molecular equivalent of NaOH. The brom thymol blue was found to be decidedly toxic upon injection into the eggs. All the other dyes were relatively non-toxic except brom cresol purple which gave evidence of toxicity only when injected into the cell nucleus. The Needhams, who used dyes from the British Drug Houses, did not find brom thymol blue to be especially toxic. On the other hand, they reported that the brom cresol purple produced cytolysis with considerable ease.

Fortunately, all the dyes in Clark's series for determining the pH are used as sodium salts and do not cause coagulation but quickly spread through the protoplasm and give it an even, diffuse color. This feature has already been noted for certain other acid dyes (21).

Neutral red, a basic dye (either the chloride or iodide of the color base), tends to coagulate protoplasm when it is injected (13, 21). If very little is introduced, the coagulating effect is localized at the spot of puncture and the dye diffuses slowly and evenly through the rest of the protoplasm. The diffuseness disappears after some time when the color accumulates in or on the cytoplasmic granules. Regions which are thickly beset with granules then appear more deeply colored than regions where the granules are sparse. In the following series of experiments the tints were recorded while the color was still in the optically homogeneous cytoplasm.

The dyes were injected both in their alkaline and acid states and in varied quantities. As long as the injection produced no visible signs of irreversible injury to the protoplasm the color always turned to that characteristic of a constant pH value. There was, therefore, no danger of masking or swamping out the cytoplasmic pH by the possible introduction of an excessive amount of the indicator solution.

The use of a completely overlapping series of indicators which show actual changes in tint rather than intensity differences were depended upon for determining the pH. Comparisons of the colors were made with the indicators in Clark and Lubs' standard buffer solutions. Direct comparisons on the stage of the microscope were also made by means of capillary glass tubes filled with the dye and by Pantin's method (22) of projecting the image of a series of colored test-tubes into the microscopic field.

The source of illumination was a 100-Watt nitrogen-filled, tungsten (Mazda C) bulb the magnified image of which was cast on the plane mirror of the microscope by means of a glass globe filled with water. Between the globe and bulb was inserted a ground "Daylite Glass," a color screen devised by Gage (23) for producing daylight artificially.

A Leitz aplanatic-achromatic, N.A. 1.40, condenser was used with its top lens removed (24). For critical reading the Leitz, 3 mm. apochromatic objective with an 8 X, periplan ocular was used.

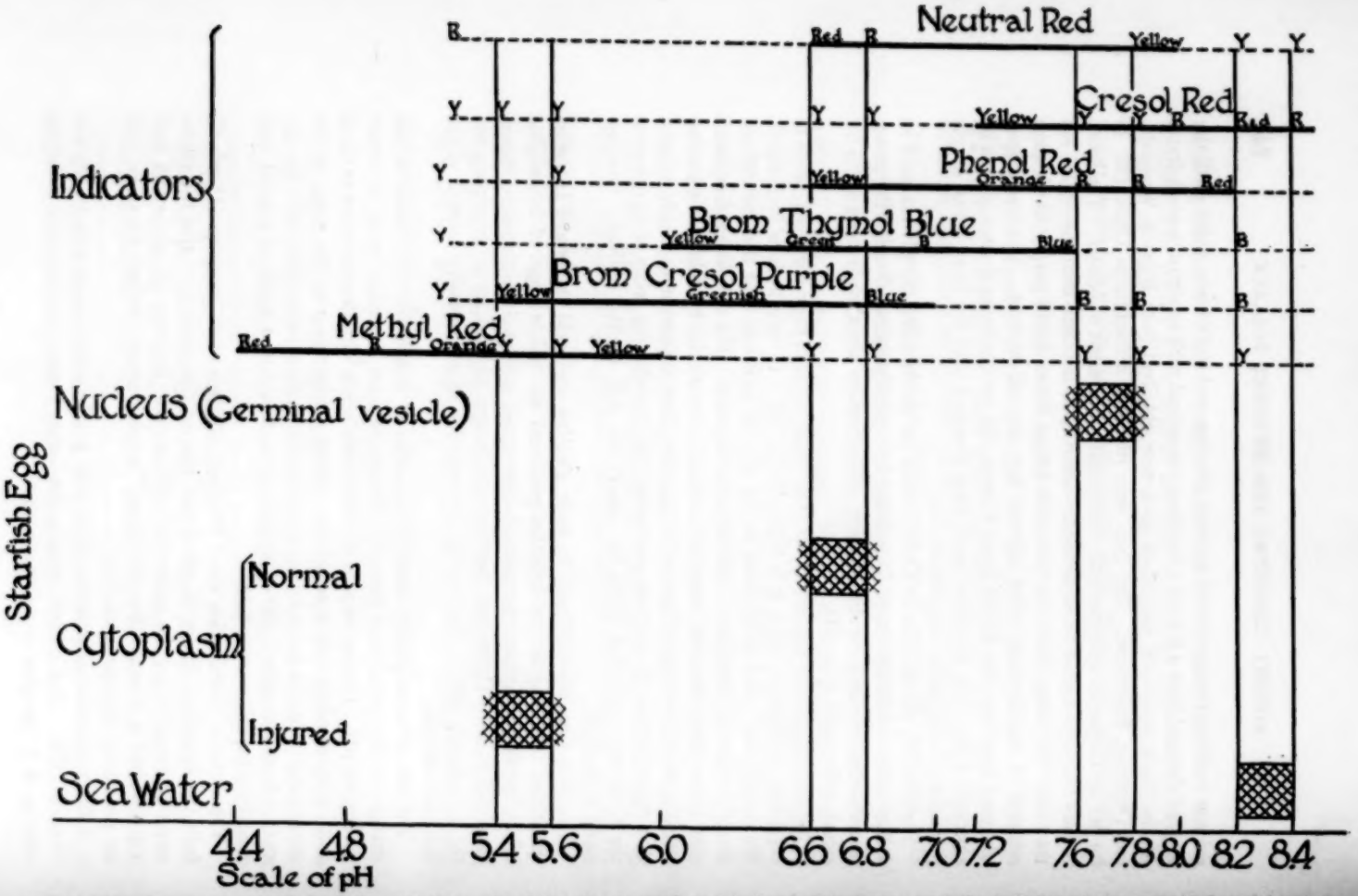


FIG. 1. Tabular representation of the pH determined for the normal and injured cytoplasm, and the interior of the germinal vesicle of the starfish egg.

B. Experiments.

1. The Cytoplasm.

The dyes, when injected into the cytoplasm of the starfish egg, give colors which indicate a pH of 6.7 ± 0.1, Fig. 1. This value was determined from the injection of phenol red which gave a yellow color with no appreciable red tinge. The true colorimetric value may be one or two decimal points above this figure owing to the fact that any tinge of red would be obscured by the faint yellow pigment present in the normal cytoplasm. The same value

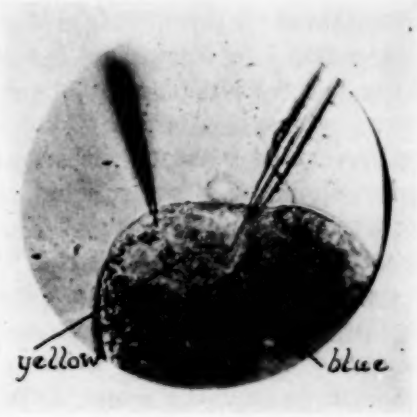


FIG. 2. Photograph of mature, unfertilized egg of the starfish injected with brom cresol purple and locally injured by a thrust of a micro needle. The region cytolyzed by the injury is yellow, the healthy cytoplasm is blue.

was obtained for the cytoplasm of eggs in the unfertilized, fertilized, and the first and second cleavage stages. Our results, therefore, closely approximate those of the Needhams.

2. Effect of Injury on the Cytoplasm.

(*a*) *Injury Accompanied by Visible Disintegration.*—A rapid tear of the cytoplasm of an egg induces cytolysis which spreads from the spot of injury (12, 13). Frequently, the spread of the cytolysis is stopped by the formation of a new membrane between the healthy and cytolyzing cytoplasm. Such a case is illustrated in Fig. 2.

FIG. 1. Tabular representation of the pH determined for the normal and injured cytoplasm, and the interior of the germinal vesicle of the starfish egg.

An unfertilized starfish egg, colored blue by injecting brom cresol purple, was injured by repeated thrusts of a micro needle. The photograph was taken after the cytolysis had been localized. The immediate change in color of the cytolyzing region from blue to yellow shows that there is a rapid production of an acid due to injury which changes the pH to 5.6 or lower. A similar treatment of an egg colored yellow with injected methyl red results in no color change. This shows, *cf.* Fig. 1, that the pH of cytolysis is between 5.4 and 5.6. The same value was obtained when eggs were cytolyzed in sea water as acid as is consistent with viable conditions (pH 6.0). The cytolyzed region keeps its pH for several minutes until the seeping in of the sea water shifts the pH to that of the surrounding medium.

A phenomenon which may be of significance in a study of cytolysis occurs if a starfish egg, injected with brom cresol purple, is injured so as to produce extensive cytolysis. The yellow, disintegrating material gradually separates into two constituents: a loose, granular coagulum, colored yellow, and an oil-like, free flowing liquid, colored blue. On standing, the latter becomes semi solid.

The acid due to mechanical injury can also be detected in the environment of the egg. This is shown in the following experiment. An immature starfish egg, immersed in sea water colored with brom cresol purple was injured with a needle. Prior to visible cytolysis of the egg, the sea water immediately around it turned yellow and, after a few seconds, reverted to the original blue color.

(*b*) *Injury Unaccompanied by Visible Disintegration.*—The mere fact that a slight tear or puncture of an egg causes no morphological changes characteristic of cytolysis does not indicate that no injury has resulted. The two following experiments offer evidence that an acid due to injury is produced with no consequent visible cytolysis when a micro pipette punctures an egg in the course of an injection or when the egg is slowly torn. A micro pipette, having an aperture of half a micron, was filled with brom cresol purple in its blue state. The pipette was then thrust into an egg and the dye immediately injected. The region of the puncture at once took on a distinctly yellow color in contrast to the blue which slowly spread throughout the rest of the cytoplasm. 1 or 2 seconds after the injection the yellow color, at the spot where the puncture had been made, changed to a

blue. In the other experiment a starfish egg, previously injected with brom cresol purple, was carefully punctured and slowly torn with a needle. A flash of yellow appeared in the immediate vicinity of the puncture quickly followed by a return to the blue.

In both of the above experiments the loss of the yellow color resulted in a disappearance of the only evidence that the cytoplasm had ever been punctured or torn.

3. The Nucleus of the Immature Egg.

The susceptibility to mechanical injury of the germinal vesicle or nucleus of the immature starfish egg has been previously demonstrated (12, 13).

By taking special precautions it was possible to insert a pipette into the germinal vesicle and to inject indicator dyes into it with no visible sign of injury. The most serviceable pipette for this purpose is one with a tip which tapers rapidly and then extends as a hollow, rigid hair 8 or 10 micra long and a little over 1 micron in diameter at its base. The aperture at the hair tip is less than half a micron in diameter. Pipettes of hard or Pyrex glass are brittle and too easily broken. Soft glass pipettes are more satisfactory and can be rendered sufficiently free of alkali for the period of the experiment by rinsing before use.

An egg was held with a micro needle against the edge of a hanging drop of sea water. The pipette was then thrust into the egg and slowly pushed against the nucleus which it indented. The tip of the pipette finally broke through the nuclear membrane without causing visible injury. After a small amount of the indicator had been injected, the pipette was slowly withdrawn and the minute puncture closed as the indentation of the nuclear membrane flattened out. The egg was then pushed into the deeper region of the hanging drop where the nucleus resumed its normal shape and appearance except for the color of the injected dye. In this way all the dyes indicated in Fig. 1 except brom thymol blue and methyl red were successfully injected. Brom thymol blue was omitted because of its toxicity and methyl red because its useful range is too low.

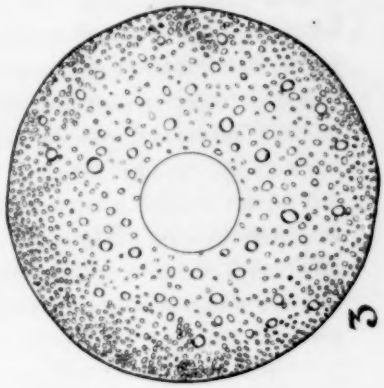
The colors assumed by the dyes indicate an intranuclear pH between 7.4 and 7.6 (*cf.* Fig. 1). Brom cresol purple, in addition to coloring

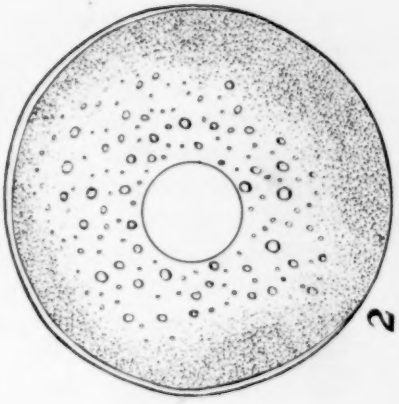
the nuclear sap blue, fixes the nucleolus and stains it an intense purplish blue. Phenol red and neutral red are the least toxic and it was after the injection of these two dyes that a maturation of the injected germinal vesicle was observed. Fig. 3 shows three photographs of an egg whose germinal vesicle was injected with phenol red. In Fig. 3, 1, the tip of the pipette can be seen at *a* in the germinal vesicle of the egg which is flattened by being brought into the shallow part of the hanging drop. After the injection the germinal vesicle was colored diffusely rose red. Some of the dye passed into the cytoplasm either directly or through the nuclear membrane. The yellow color of the cytoplasm and the red of the nucleus offered a striking contrast. Fig. 3, 2, is a photograph of the egg 1 hour later when it had been returned to the deeper region of the hanging drop. The onset of a typical maturation is to be noted. The germinal vesicle has begun to collapse and its membrane to wrinkle and fade. The red nuclear sap streamed in several radial paths into the yellow cytoplasm which took on an everdeepening orange tint. After several minutes the orange color changed back to the original yellow. The last photograph, 3, was taken 1 hour later and shows the diminutive pronucleus in the state which precedes polar body formation. In the four cases in which this phenomenon was observed (three after the injection of phenol red and one after that of neutral red) no polar bodies were formed.

FIG. 3. Photograph of starfish egg undergoing maturation with its cytoplasm and germinal vesicle injected with phenol red. 1. Immature egg held by needle "*b*" in shallow region of hanging drop and with micro pipette "*a*" vertically inserted into the germinal vesicle. The cytoplasm is yellow, the germinal vesicle is red. 2. Egg, 1 hour later, in deeper region of hanging drop. The germinal vesicle has begun to shrivel and the cytoplasm is taking on an orange tint. 3. Egg 10 minutes later, with yellow cytoplasm. The diminutive pronucleus prior to polar body formation can be seen in the center of the egg.

FIG. 4. Sketches to show effect of mechanically injuring the germinal vesicle of a starfish egg. 1. Before injury. 2. Immediately after injuring the egg either by crushing or by puncturing the germinal vesicle. The remains of the germinal vesicle is to be seen as a hyaline sphere (nuclear remnant) and the cytoplasm around it has cytolyzed. The vitelline membrane is partially lifted, a phenomenon which frequently occurs when an egg is injured, *cf.* Chambers (12). 3. Completely cytolyzed egg within vitelline membrane.

FIG. 3





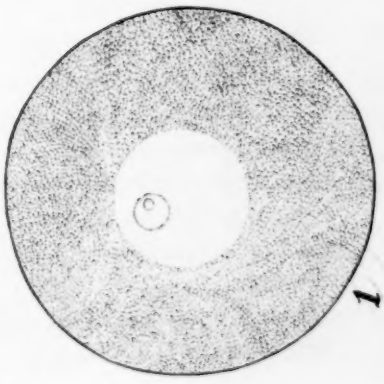


FIG. 4

The uniform orange tint in the germinal vesicle after an injection of neutral red was followed almost immediately by a deep, red zone in the bordering cytoplasm. The color in the nucleus rapidly became paler and almost completely faded while the cytoplasm tinged a rose red. Neutral red was the only dye which faded from the nucleus to such an appreciable extent.

The nucleolus is a more or less solid body and tends to become more intensely colored in time than the rest of the nucleus. When a stream of neutral red is directed against the nucleolus, the color of the dye spreads slowly through it from one side.

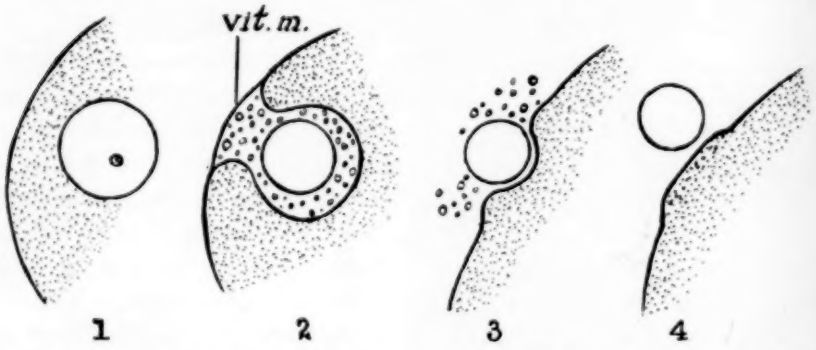


FIG. 5. Injury of germinal vesicle followed by extrusion of nuclear remnant. 1. Before injury. 2. Nuclear remnant in cytolyzed region which is walled off from healthy cytoplasm. vit. m. = vitelline membrane. 3 and 4. Nuclear remnant which is being extruded after breakdown of vitelline membrane.

4. Effect of Injury on the Germinal Vesicle.

The usual sign of approaching disruption is the fading of the nucleolus. This is followed by a cytolysis which spreads from the surface of the injured nucleus. A spherical, optically homogeneous, nuclear remnant frequently persists in the disintegrated region, Fig. 4, and can be dragged out into the surrounding sea water. Brom cresol purple has the peculiar property of frequently fixing both the nucleolus and the nuclear membrane without hindering disintegration of the rest of the egg.

If an injected germinal vesicle is injured there is no change in color regardless of the indicator used. This is in striking contrast to the

immediate change which is produced in the cytoplasm upon injury. The germinal vesicle and cytoplasm of a starfish egg, Fig. 5, 1, were injected with phenol red and injured so as to produce cytolysis, Fig. 5, 2. The healthy cytoplasm and the débris resulting from cytolysis were yellow and the nuclear remnant red. In Fig. 5; 3 and 4, the healthy egg remnant has rounded up and the consequent flattening out of the bay has carried the nuclear remnant and the cytolyzed débris into the surrounding sea water. The débris turned red on coming into contact with the sea water the pH of which is 8.4. The nuclear remnant maintained its red color for several seconds until it collapsed and disappeared. This shows that injury to the nucleus causes no increase in acidity.

Cresol red was injected into the germinal vesicle of another egg. Upon injury the nuclear remnant retained the yellow color until it collapsed in the cytolyzed débris. This shows that injury to the nucleus causes no increase in alkalinity.

The ease with which the nuclear remnant assumes the color of its environing medium is seen from the following. If brought into sea water colored with phenol red it turns red if the sea water has a pH of 8.4 and yellow if it has a pH of 6.0.

A germinal vesicle was injected with brom cresol purple. Upon injury to the nucleus, extensive cytolysis of the egg ensued. The acid débris and the blue nuclear remnant were retained within the confines of the persisting vitelline membrane. The nuclear remnant gradually changed from a blue to a yellow color, indicating that it had assumed the pH of its immediate environment of cytolyzed material.

5. The Rate of Surface Membrane Formation in Its Relation to the Entrance of Dyes through a Torn Surface of a Starfish Egg.

Frequently, if cytolysis occurs when too large a puncture is made in injecting a plasmalemma quickly forms about the cytolyzed area and the fluid ejected from the pipette simply lies in a pocket sharply marked off from the healthy cytoplasm. When this occurs with eggs immersed in normal sea water, the newly formed membrane serves as an effective barrier against the passage of the dye into the cytoplasm. Evidently, the membrane must form with extreme rapidity. If, however, the same procedure is carried out on eggs in sea

water having a pH of 6.0, the dye frequently penetrates into the cytoplasm. This indicates that this new surface membrane in an environment which is more acid than normal, either has a different permeability, or is retarded long enough in its formation to allow the dye to enter. It is possible that both factors are operative.

DISCUSSION.

Of the considerable number of papers which have recently been published on intracellular pH, only those can be specially mentioned

TABLE I.

Differences in Dissociation of Acid and of Basic Dyes in Their Acid and Alkaline Ranges.

Dyes		Acid range	Alkaline range
Acidic	Brom cresol purple	Low dissociation Yellow	High dissociation with salt formation **Blue***
	Phenol red	Low dissociation **Yellow***	High dissociation with salt formation Red
Basic	Methyl red	High dissociation with salt formation Red	Low dissociation **Yellow***
	Neutral red	High dissociation with salt formation **Red***	Low dissociation Yellow

* Color assumed by the cytoplasm when the dye is injected into it.

which deal with marine ova. Our results on the pH of normal cytoplasm of starfish eggs are in close accord with those of the Needhams. We place the pH of normal cytoplasm of the eggs between 6.6 and 6.8, the Needhams place it at 6.6 ± 0.1.

In answer to the possible objection that the errors are too great to permit a determination of the protoplasmic pH, it may be pointed out that all the dyes give consistent indications toward the same pH irrespective of their chemical constitution, Table I. For ex-

ample, brom cresol purple gives to the cytoplasm the blue color of the salt of its alkaline range while phenol red imparts to the cytoplasm the yellow, non-dissociated color of its acid range. The same principle also holds true for the two basic dyes used: *viz.*, neutral red and methyl red.[2] Both are yellow in their alkaline ranges where they are in the state of lowest dissociation and least salt formation. In their acid ranges, where their dissociation is greatest and, consequently, where salt formation predominates, both of the dyes are red in color. The fact that methyl red gives a yellow while neutral red a rose red color when injected into the cytoplasm is added evidence that the hydrogen ion concentration is a prime factor in the formation of the colors.

The production of an acid associated with injury or death of cellular tissues has been frequently reported in the literature (3, 4, 19, 25).

Concerning the pH of the injured cytoplasm of echinoderm eggs our results differ somewhat from that of the Needhams. In the eggs of *Paracentrotus lividus* they placed the value below 5.0 and above 4.0 because of the results obtained with methyl red and brom phenol blue. The methyl red we used was the sodium salt while they used the saturated aqueous solution.

The difference in reaction of the egg to a slow and to a rapid tear is probably due to the amount of acid produced by the injury. With a slow tear very little acid results at any given moment and it is presumably neutralized as fast as it is formed. With a rapid tear a considerable amount of acid is produced which cannot be taken care of by the cytoplasmic buffers upon which cytolysis sets in. With the spread of the disintegration more acid accumulates and the cytolysis continues. It is also significant that mechanical injury occasions an increase in acidity both outside the egg and within its cytoplasm, before there is any visible sign of cytolysis.

In this regard it is of interest to note the pH findings on echinoderm eggs by Vlès and his coworkers. They crush the eggs in the indicator and observe the resulting color. This method is open to several objections: first, there is the danger of mixing the intracellular fluids

[2] Methyl red has both an active acid and basic group in its molecular structure. The indicator is generally used as the sodium salt, but it shows the typical dissociation curve of a base. It is the basic group which is responsible for the color changes from red to yellow.

with the fluid which surrounds the cells; second, the cytoplasmic fluid may also mix with the fluids of intracellular vacuoles; and, third, the injury to the plasma membrane, which is a necessary consequence of crushing cells, almost always initiates disintegrative changes in the protoplasm. In addition to this the instantaneous production of an acid upon mechanical injury followed by the further development of acid concomitant with visible cytolysis must, to a considerable degree, modify the actual pH of normal, uninjured cytoplasm.[3]

After the publication of the Needhams' criticism of the crushing method Vellinger (27) checked the previous potentiometric determinations (9) of egg material procured by crushing the eggs in a chamber cooled to −60°C. Potentiometric readings were then made on the powder as it thawed. The first readings gave the highest pH. Subsequently, as the temperature rose, the pH dropped until it reached a constant value equal to that already recorded by Vlès, Reiss, and Vellinger (9) as the normal pH of the cytoplasm of the eggs. The fact that Vellinger's first readings give the highest pH, can be interpreted to mean that the excessively low temperature prevents or at least delays the production of the acid accompanying injury when the eggs are crushed. The first readings should then more nearly approach the pH of the normal cytoplasm. With the progressive thawing of the egg material more and more acid is produced and hence the pH falls till it reaches a level typical for cytolysis.

In this connection may be mentioned the recent result of Bodine (28) who obtained some fluid from the large yolk-laden *Fundulus* egg by pricking the dried surface of the egg. The exuding fluid was drawn into a dry glass capillary. The pH of the fluid, determined potentiometrically, was found to be 6.4.

The Needhams made no special investigation of the pH of the germinal vesicle but it is significant that they report it to give the alkaline color of brom cresol purple in both the *Echinocardium* and the *Asterias* egg even after cytolysis had occurred.

[3] Reiss (26) claims to have found by his crushing method that the pH of the *Paracentrotus* egg changes during the different stages of its development. The values which he gives lie between the extremes of 5.3 and 5.6 and are small enough to be considered within the limits of probable error. However, it is conceivable that difference in pH may occur in the disintegrated material obtained from cells in the different stages of their development.

The fact that the nucleus does not change in reaction after cytolysis of the egg, indicates why Reiss was able to report from his results on eggs crushed in indicator solutions that the nucleus is faintly alkaline. This is in accordance with the results we obtained with the nuclear remnants of crushed *Asterias* eggs.

In order, however, to determine the pH of the normal nucleus there must be a definite proof, as given in the experimental part of this paper, that both the egg and its nucleus are alive and active during the period of the determination.

The difference in the hydrogen ion concentration between the nucleus and cytoplasm of the living immature starfish egg is of considerable interest. It would, however, be premature to speculate from this on the interrelationships of the nucleus and cytoplasm of cells in general. On the other hand, it might well be pointed out that the immature egg, although it has a much enlarged nucleus, is, nevertheless, more truly to be compared with a somatic cell than the mature egg.

SUMMARY.

I. *Cytoplasm.*

1. The normal cytoplasmic pH, colorimetrically determined, of the starfish eggs in the unfertilized, fertilized, and first and second cleavage stages is 6.7 ± 0.1.

2. Cytolysis lowers the pH to a value 5.5 ± 0.1.

3. The cytolyzed material in time assumes the pH of its environing sea water.

4. The acid due to mechanical injury can also be detected in the environment of the egg.

5. Injury to the cytoplasm unaccompanied by visible disintegration causes an increase in acidity which is quickly neutralized.

II. *Germinal Vesicle.*

6. The intranuclear pH, colorimetrically determined, of the immature *Asterias* egg is 7.5 ± 0.1.

7. Injury to the nucleus does not change its pH.

8. The spherical nuclear remnant which persists after injury gradually assumes the pH of its environment.

III. *Plasmalemma.*

9. A dye to which the cell is normally impermeable can penetrate through a tear in the surface from an environment more acid than normal. This may be due to a difference in the formation of the plasmalemma in a normal and an acid medium.

We take this opportunity of thanking Dr. Barnett Cohen of the Hygienic Laboratory, Washington, for valuable advice given in the preparation of this paper.

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GEOTROPISM OF AGRIOLIMAX.

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I.

Analysis of the geotropic conduct of young rats (Crozier and Pincus, 1926–27, *a*, *b;* Pincus, 1926–27) showed that it is possible to describe the negative geotropism of these animals by simple mathematical expressions. The results gotten from mammals may be shown to have a general value, as soon as it becomes possible to find similar relationships with invertebrates.

The negative geotropism of gastropods, as in *Helix* (Cole, 1925), *Limax* (Crozier and Federighi, 1924–25; Davenport and Perkins, 1897–98), and other forms (Kanda, 1916) suggested that such animals could be used for an exact investigation of their geotropic conduct. The common garden slug *Agriolimax lævis campestris* (Binney) was available during fall and winter months, and was used for these experiments.

II.

A large number of animals was kept for several weeks in the dark room, where the experiments were made. During an experiment several individuals were put on an inclined plate of ground glass, which was moistened every few minutes. In darkness the slugs creep almost always upward, and it was interesting to see whether we could find here in the same way an increase of the angle of orientation if the angle of the creeping plane with the horizontal was increased, as in the experiments with the young rats. The angle of inclination of the glass plate was varied between 45° and 90°. As soon as the animals were put on the plate they started to creep and described a certain path which was observed every few minutes with a red flash-

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light. The plate was divided in squares so that the path made during the interval could be copied on a coordinate paper on which the angles of orientation were subsequently measured. In most cases the animals creep upward; downward creeping occurred only seldom. In general, only the angles of upward orientation were taken into

TABLE I.

Angles of orientation (Θ) of *Agriolimax* at an inclination of the creeping plane at angle (α) 51° to the horizontal, during upward and during downward orientation. In both directions the mean angle of orientation is the same.

Values for (Θ) during	
Upward orientation, 58°, 58°, 60°, 58°, 53°, 55°, 58°, 56°, 62°, 55°, 55°, 61°, 57°, 61°, 54°	mean 57.4°
Downward orientation, 61°, 62°, 53°, 52°, 54°, 58°, 55°, 50°, 54°, 62°, 58°, 58°, 52°, 63°, 55°	mean 56.4°

TABLE II.

Mean angles of orientation (Θ) of *Agriolimax* during creeping upon a surface inclined at different angles (α) with the horizontal; the probable error of each mean angle; and the coefficient of variation of Θ.

α	Θ	P. E.	C. V.
45°	48.7°	±0.72	11.0
51°	55.9°	±0.66	13.2
55°	61.8°	±0.59	7.16
60°	69.3°	±0.66	10.6
66°	72.3°	±0.46	5.27
72°	79.8°	±0.38	3.64
79°	84.1°	±0.41	3.91
90°	88.7°	±0.28	2.39
108°	77.4°	±0.58	6.08
120°	69.9°	±0.47	5.59
129°	56.5°	±0.92	13.6

account, but on comparing these with the angles described during downward movement, almost no difference was found. The animals describe in either direction the same angle Θ (Table I). Furthermore, it is to be said that very often the direction of the path becomes changed, the animals turning to the right or to the left, but the

angle (Θ) remains also in both cases the same. It is important, however, to say that when the slugs stop creeping no definite angle corresponding to the measured angle of orientation was observed.

The results summarized in Table II are averages of from 30 to 50 tests at each angle of inclination of the creeping plane. For the experiments the following angles were chosen: 45°, 51°, 55°, 60°, 66°, 72°, 79°, 90°, 108°, 120°, 129°. Several tests with angles below 45° gave indecisive results; the animals describe more or less circles, no definite direction being adhered to; the mean of the single tests falls always near 45° orientation (Θ), which shows that the deviation from the angle which should supposedly be taken is rather great. It seems that in all these cases the downward pull becomes so small that the animals do not orient as in the cases where higher angles were used. Some of the experiments were done during the fall of 1926; it seemed interesting, however, to control the results by later tests. For the repetition the angles 51°, 60°, and 72°, together with the angles, 108°, 120°, and 129° were taken. The difference between the mean values of Θ and those obtained a few months before was not more than 2° or 3°. The reproducibility of the angle Θ therefore seems little influenced by season, by weight of animal, or by speed of creeping. The tests with angles above 90° were interesting as showing whether there is an influence of the statocysts on the angle of orientation, because the animals are now hanging from the creeping plane. The difference between the angles at inclinations below and above 90° is, however, so small that it seems that the angle of orientation is not controlled by the statocysts.

Table II gives the results of the experiments: (1) the angle of inclination (α) of the glass plate to the horizontal; (2) the angle of orientation in the creeping plane (Θ), with its P.E.; (3); and (4) *C.V.*, the measure of the variability of Θ.

The degree of upward orientation (Θ) increases with the increase of the inclination of the creeping plane (α). The probable error is rather small in every case and it decreases with the increase of α.

As in the experiments with the young rats, it can be shown that the angle of orientation (Θ) is proportional to the logarithm of the sine of the angle in the creeping plane:

$$\Theta = k \log \sin \alpha + C.$$

The graph in Fig. 1 gives a picture of this relationship. The points fall almost altogether on a straight line, aside from the two points for the observation with $\alpha = 45°$ and $\alpha = 90°$. When the creeping plane is inclined at about 45° it seems that the total downward pull is too small, so that the lower limit of geotropic excitation is reached, and the animals begin to show frequent circus movements as during the experiments with still lower angles. In case the creeping plane is vertical, the animals seldom creep straight up; one gets always a

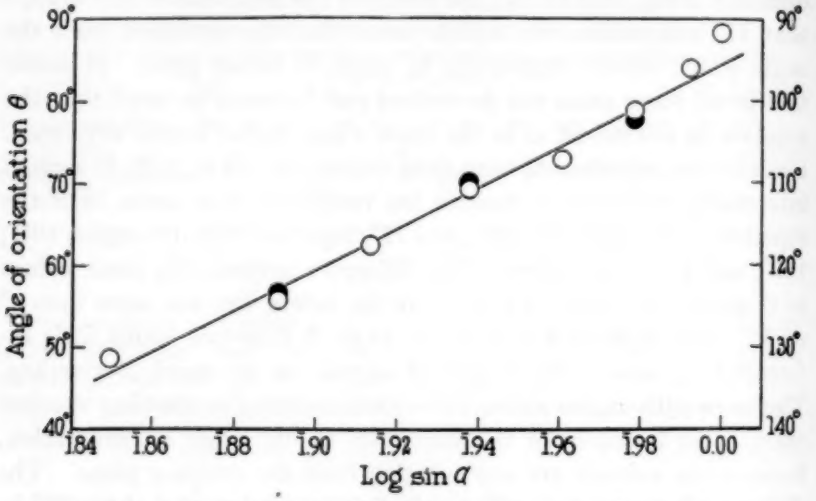

FIG. 1. The angle of orientation (θ) is directly proportional to *log sin* α, where α is the angle of inclination of the creeping plane. The points are averages of 30 to 50 measurements each. The solid circles are from measurements made at inclinations greater than 90° (*i.e.* the animal hanging from the under surface of the plane).

little deviation to the right or to the left. Measuring the angles, we get up to 5° deviation from the vertical; but for simplification only angles between 85° and 90° were recorded, not between 90° and 95°; this explains why Θ, at $\alpha = 90°$, must appear to be a little less than 90°.

The line fits the points gotten in these experiments where the animals were in the upper side of the glass plate, and fits equally well the points for the angles (α) beyond 90° where the animals were hanging from the under side of the plate.

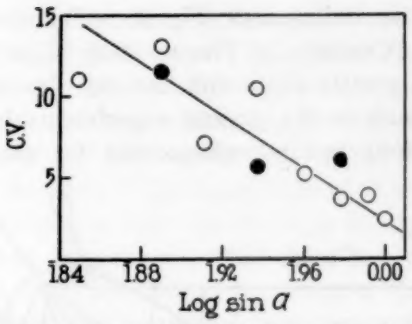

FIG. 2. The coefficient of variability of the angle of orientation decreases steadily with the increase of the logarithm of the sine of the angle of inclination of the creeping plane (see text).

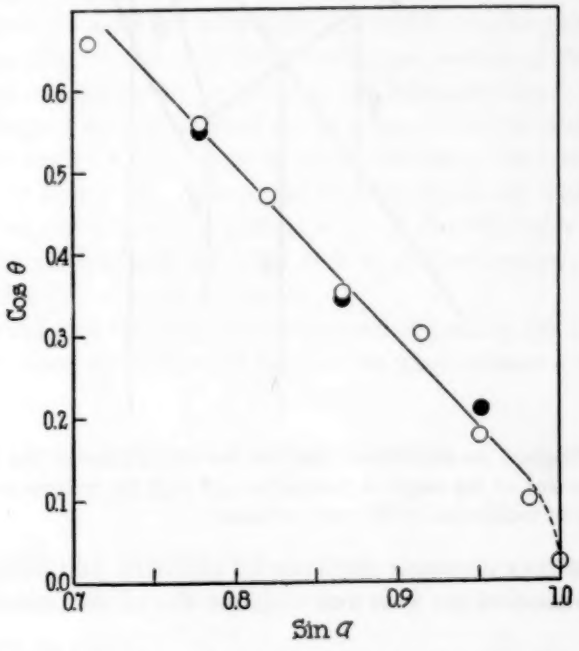

FIG. 3. The cosine of the angle of orientation (θ) decreases almost in direct proportion to the sine of the angle of inclination (α) of the creeping plane.

The coefficient of variability of the measured angles of orientation, which measures inversely the precision of orientation, decreases

steadily as *log sin* α increases (Fig. 2). This corresponds to the results with rats (Crozier and Pincus, 1926–27, *a*, *b*). The scatter of the figures is greater than with the rat observations, probably because the animals in the present experiments form a less homogeneous population; factors unimportant for the *mean extent* of

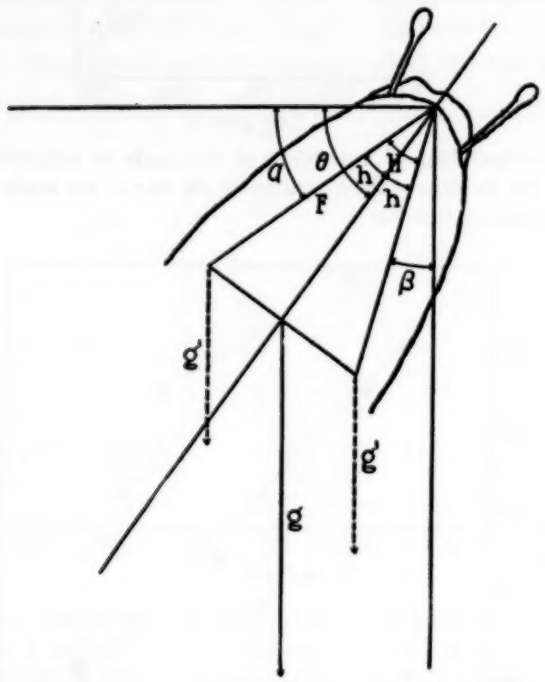

FIG. 4. Diagram showing terms used for the explanation of the relationship between the sine of the angle of orientation (θ) and the reciprocal of the sine of the angle of inclination of the creeping plane.

orientation may obviously influence its precision, and probably vary with the season of the year and with the size of the individual.

III.

After having gotten the logarithmic relationship between the pull of gravity and the extent of the orienting response of the animals, the question arises, whether for the slugs, as in the experiments with the young rats, the cosine of the angle of upward orientation (θ)

decreases directly in proportion to the sine of the angle of the inclination of the creeping plane (α). Fig. 3 shows that for *Agriolimax* we apparently find the same relationship, at least quite nearly.

In the case of the rats the explanation for this was given on the basis of the different extension of the legs on the "up" and on the "down" sides of the body. The magnitude of Θ was dependent on the differential pull on the legs. In the slugs an explanation of this kind could be given by assuming that there is a difference in the pull of the weight or a difference in the stress of the muscles of the two sides of the body; but neither of these assumptions can lead to a satisfactory result, because we have in neither case the possibility to get a quantitative expression for the distribution of the weight or of the muscle tensions. Nevertheless, it can be shown that by plotting *cos* Θ against *sin* α we get a straight line which fits the points of the observation (Fig. 3). A very much better explanation of the conduct of the slugs can, however, be given in the following way.

In the slug the muscle fibers of the anterior end of the body diverge at a certain angle (H); the angle between the axis of the body and the body wall is $H/2 = h$. The angle between the body wall and the horizontal on one side of the animal $= \alpha$, on the other side the angle between the vertical and the body wall $= \beta$; furthermore, the angle of orientation $(\Theta) = \alpha + h$ (Fig. 4).

On both sides of the slug the component of gravity (g) acts in the same way; when orientation is reached we may assume (*cf.* Fig. 4) that

$$g'x\,[\cos\alpha - \cos(\alpha + H)] \text{ becomes constant;}$$

$$\therefore \cos\alpha - \cos(\alpha + H) = \frac{K}{g},$$

$$\text{or } \cos(\Theta - h) - \cos(\Theta + h) = \frac{K}{g},$$

$$= \cos\Theta\cos h + \sin\Theta\sin h - \cos\Theta\cos h + \sin\Theta\sin h,$$

$$\text{and } \sin\Theta\sin h = \frac{K'}{g},$$

$$\sin\Theta\sin\alpha = \frac{K''}{\sin h} = \text{constant},$$

$$\therefore \sin\Theta = \frac{K''}{\sin\alpha}.$$

As shown in Fig. 5, the agreement of the observations with this equation is better than in the case of the cosine formula. The explanation here adopted, while slightly different from that used for the rats (Crozier and Pincus, 1926–27, *a*, *b*) in reality turns upon exactly the same sort of effect, namely the distribution of the pull of the animal's weight upon the two sides of the body. The fact that the cosine formula is almost obeyed (Fig. 3) is due to the fact that $1/sin\ \Theta$ is very nearly proportional, for angles not too small, to $cos\ \Theta$.

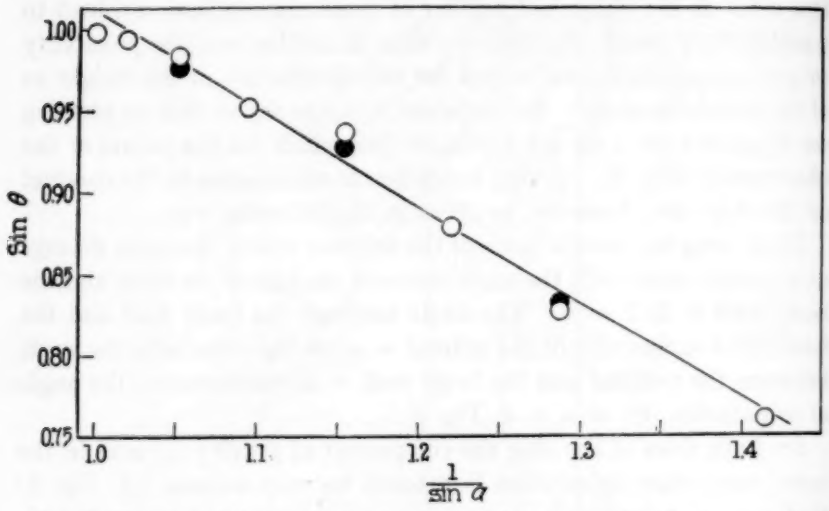

FIG. 5. The sine of the angle of orientation (Θ) decreases in direct proportion to the reciprocal of the sine of the angle of inclination of the creeping plane.

Against this explanation it might be said that perhaps the cause for taking a certain angle (Θ) at orientation is not dependent upon differences of pull on the two sides, or stress, or the head angle, but upon the pull working on the eyestalks. Several tests, however, with animals where the eyestalks were removed showed that they have no influence on the angle of orientation; the slugs describe, if they creep at all, the same angle (Θ) as when the eyestalks are present.

SUMMARY.

On an inclined glass plate the slug *Agriolimax* orients and creeps upward or downward. The angle of orientation on the plane (θ) is proportional to the logarithm of the component of gravity in the creeping plane. The coefficient of variability of the measured values of (θ) decreases linearly as the logarithm at the gravity component in the creeping plane increases. The cosine of the angle of orientation decreases almost directly in proportion to the sine of the angle of inclination of the creeping plane to the horizontal, as previously found for young rats (Crozier and Pincus). But a more satisfactory formulation for the present case shows that the sine of the angle of orientation (θ) decreases in direct proportion to the increase of the reciprocal of the sine of the angle of inclination of the creeping plane. This formulation is derived from the theory that the geotropic orientation is limited by the threshold difference between the pull of the body mass on the mutually inclined longitudinal muscles at the anterior end of the slug.

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THE OXYGEN CONSUMPTION OF FROG NERVE DURING STIMULATION.

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For many years the measurement of the possible increase in the oxygen consumption of nerves during activity has been a problem of serious difficulty. During last summer at Woods Hole I was able to overcome this difficulty by making use of the large nerves of the dogfish (following in this the lead of Parker (1925, *a*)) and by at the same time modifying Thunberg's micro respirometer so as to increase its sensitivity. In this preliminary work with the dogfish (Fenn, 1927) it was possible to demonstrate a sharp increase in the oxygen consumption at the beginning of stimulation, persisting for some time after the close of stimulation, but returning to the original rate within about 30 minutes or less. The excess oxygen taken in during activity was found on the average to be equal to 0.21 c.mm. per gm. of nerve per minute of stimulation, the resting rate being 1.35 c.mm. per gm. per minute. The absolute value of this excess oxygen is about 3 or 4 times too small to account for the heat production of nerve during stimulation as found by Downing, Gerard, and Hill (1926) in the frog nerve.

For purposes of comparison with these heat values it was important to obtain similar measurement of oxygen consumption on the frog nerve. These nerves being smaller, there was less likelihood of the oxygen tension being reduced to zero in the interior of the nerve trunk and hence a possibility of obtaining a greater excess oxygen consumption during stimulation. To improve matters still further I have studied the frog nerves in an atmosphere of oxygen, which was not easily available to me at Woods Hole. In spite of these modifications the figures now available for frog nerve are little larger than those obtained with the dogfish nerve, and the discrepancy

between the values for oxygen and that for heat remains. At the present time the most obvious difference to which this discrepancy might be ascribed is the much greater duration of stimulation (20 to 30 minutes) necessary for determinations of oxygen as compared to the 10 seconds stimulation which suffices for heat measurements.

Method.

The method which I have used for frog nerve is the same as that previously described for dogfish nerve, with few modifications. The apparatus consists essentially of two 12 cc. bottles connected by a very fine capillary which carries a kerosene index drop. The nerve is laid on the stopper of one of the bottles in contact with sealed-in platinum electrodes. The other bottle serves for temperature compensation. Even so, a thermostat constant to less than 0.01°C. is necessary for accurate work, because chance currents in the bath affect the bottles independently. The temperature used was 22°C. throughout. Sodium hydroxide (M/4) is placed in each bottle to absorb carbon dioxide, so that the index drop, in response to the consumption of oxygen, will move toward the bottle containing the nerve. Positions of the drop are read at frequent intervals by means of a hand lens and two scales. One scale is just under the capillary the other 3 inches above it. In making a reading corresponding points on these two scales are kept in line, thus avoiding parallax. The capillary itself, with the lower scale, is under water. Further details of procedure may be found in the previous report.

The one difficulty encountered in using frog nerves in place of dogfish nerves is their small size. On the average the dogfish nerves weighed 5 times as much as those to be found in a good sized frog (30–40 mg. each). With the same apparatus, therefore, it should be possible to make corresponding measurements on four frog sciatic nerves and this turns out to be the case (Fig. 1, *a*). To avoid the labor of dissecting so many nerves it seemed worth while to try to make the apparatus still more sensitive. The instrument used for most of the measurements to be reported in this paper does not differ in general plan from the one previously described but it is smaller throughout. The bottles hold only 3.7 cc. instead of 12 cc. and the capillary (a piece of thermometer tubing) holds only 0.73 c.mm.

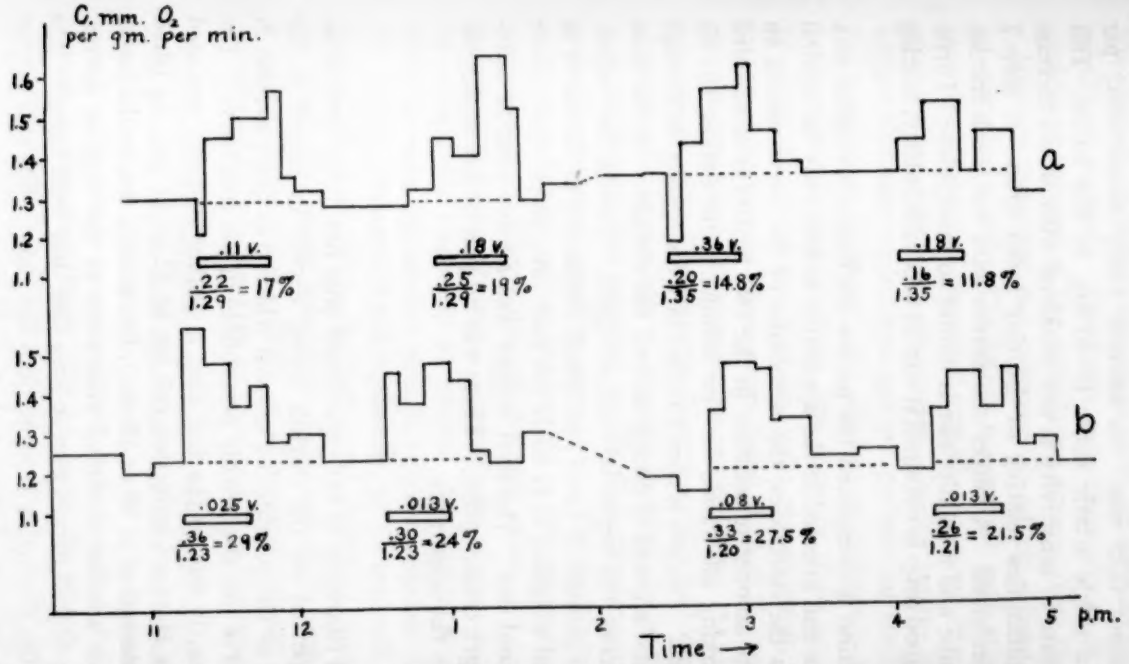

FIG. 1. Graphs showing the rate of oxygen consumption of frog nerves at rest and during stimulation periods of varying intensity. On the graph are inserted figures showing the equivalent voltage of the stimulating current derived from the secondary of an induction coil, and the percentage increase in the oxygen consumption for each period of stimulation. The total excess oxygen for each period of stimulation is represented by the area of the rise as plotted and seems to be independent of the intensity of stimulation within considerable limits.

per cm. instead of 1.94 as before. With this apparatus the index drop moves about 0.25 mm. (an amount easily measurable) per minute with a single sciatic nerve of 40 mg. in the bottle This threefold increase in sensitivity is not obtained without an increase in technical difficulties relating to the tiny index drop. A drop 2 mm. long is preferred. A longer one moves with too much friction in the small tube and a smaller one is harder to manipulate. Fortunately once a good drop is obtained it can be used indefinitely, barring accidents.

After dissection is completed the nerves are quickly weighed on a torsion balance and inserted in the apparatus so that only the central ends lie across the electrodes, the remainder of the nerve resting on the glass. The amount of solution in the two bottles is so adjusted that, after making allowance for the volume of the nerves, the air spaces are equal. Oxygen is then bubbled into the apparatus through the side arm and allowed to escape around the stopper. At the close of the experiment the nerve is weighed again. During the experiment a certain amount of blood and lymph drains out of the nerve so that the second weighing is 11 to 22 per cent (av., 16 per cent) lower than the original one. The final weight was always used in calculating the oxygen consumption. There was no perceptible drying out of the nerve in the apparatus.

Results.

The general character of the experiment does not differ from those previously reported on the dogfish nerve, except in regard to the heating effect of the stimulating current which was quite evident in the dogfish nerve but not usually perceptible in the frog nerve. The difference is partly due to the fact that a weaker stimulus was used for the frog (a Harvard induction coil set at 12 or 13 cm. (or tilted at an angle) instead of at 10 or 11 cm.), but mostly due to the larger size and hence smaller electrical resistance of the dogfish nerves. Moreover, the dogfish nerves were so long that they were brought into contact with the electrodes at more than one point, thus still further increasing the amount of current that could flow.

Intensity of Stimulation.—The results of two experiments with stimulation of varying intensity have been plotted in Fig. 1, *a* and *b*.

For *a*, four frog nerves were used in the dogfish-nerve apparatus. The stimulation periods are indicated by rectangular blocks and lasted usually 30 minutes. The figure over these blocks is the estimated equivalent voltage[1] of the stimulating current from the secondary of a Harvard induction coil vibrating at 50 per second and operated by a single dry cell. The figures underneath each stimulation block show the amount of increased oxygen measured in c.mm. per gm. of nerve per minute of stimulation, divided by the resting rate of oxygen consumption in c.mm. per gm. per minute, the quotient giving the percentage increase. No significant difference is to be seen between stimulation at 9 cm. coil distance (0.36 eq. volts) and at 10 or 11 cm., (0.18 and 0.11 eq. volts respectively), the figures for increased oxygen in these cases being 0.22, 0.25, and 0.20 c.mm. per gm. per minute of stimulation respectively. At the close of the experiment, 6 to 7 hours after dissection, this had dropped to 0.16, indicating perhaps a loss of function in some of the fibers.

In the experiment recorded in Fig. 1, *b*, two frog nerves were observed in the more sensitive apparatus. The nerves were stimulated with an induction coil as before, but a 100 per second tuning fork was used as an interrupter, there being about 0.5 volts across the primary coil during contact. The intensity of stimulation was varied by moving the secondary as before. No significant difference was found between stimulation with 11 cm. coil distance (0.08 eq. volts), 13 cm. coil distance (0.024 eq. volts), or 13 cm. coil distance with the coil tilted at 45° to the horizontal (0.013 eq. volts), the figures for these three cases being 0.36, 0.30, and 0.33 c.mm. per gm. per minute of stimulation. With an equivalent voltage of 0.013 volts the stimulus is too weak to be preceptible to the tongue, but never-

[1] The equivalent voltage corresponding to various settings of the secondary of the instrument used for these experiments has been determined by means of a thermal converter and a sensitive galvanometer. The equivalent voltage E is defined as the voltage necessary to force a direct current of the same strength through the secondary circuit. The P.D. across the primary terminals was 1 volt. The values of E for coil positions 13, 12, 11, 10, and 9 cm. were respectively 0.034, 0.055, 0.09, 0.15, and 0.3 volts. A heating effect due to high frequency radio waves direct from the spark gap without any electrical contact to the secondary has been allowed for.

theless strong enough (at least the break shocks) to produce a maximal contraction of a frog sciatic-gastrocnemius preparation. Here again at the close of the experiment a somewhat smaller figure, 0.26 c.mm., was obtained with the weakest stimulation. The irregularities in these curves represent the experimental error of the method and are due to errors in reading the position of the drop and to small irregularities in its movement. The dotted line under each rise due to stimulation represents the base line which was used in calculating the magnitude of the increased oxygen usage. In the selection of this base line there is some uncertainty. The break in the graph in Fig. 1, *a*, shown by the dotted line, represents the time necessary to move the drop back to the other end of the capillary tube. This procedure frequently upsets the reading for a short time. A similar break in Fig. 1, *b*, is of similar significance but is longer because of some technical difficulties with the index drop. No systematic attempt has been made, beyond the experiments of Fig. 1, *a*, to measure accurately the effect of varying intensity of stimulation. The experiments here reported indicate that the differences, if they exist, are not large. This indeed is to be expected from the all-or-none law and is indirect evidence that the oxygen consumption increases here observed are actually due to nerve impulses and not to electrical or other artefacts at the electrodes.

It is quite certain that curves like those of Fig. 1 cannot be obtained from bits of cotton soaked in bicarbonate solution and laid across the electrodes, nor are they obtained from dead nerves. In one experiment, for example, the negative variation and the oxygen used were being recorded simultaneously. Stimulation produced no change in either. Because of a misunderstanding the silver electrodes used for recording the negative variation had not been washed free from the strong salt solution after plating; this oversight killed the nerve and unexpectedly afforded a clean-cut control experiment.

Frequency of Stimulation.

By stimulating the nerves at varying frequencies some data have been obtained which afford a fairly satisfactory proof that the extra oxygen is actually related to the energy requirements of the nerve impulse. For this purpose tuning forks vibrating at 100 and at

50 per second were used to interrupt the primary current. Thus 200 and 100 shocks per second were delivered to the nerve through the secondary coil. In Fig. 2 there are plotted two frequency curves to show the distribution of the results obtained with 32 stimulation periods at 100 interruptions per second and 21 periods with 50 interruptions per second. Comparing the averages of these results it is evident that doubling the number of impulses per second does not double the amount of extra oxygen used but increases it only 0.315/0.268 or 1.18 times. A similar result was obtained in one experiment in which the responses of the same nerve to the two rates of

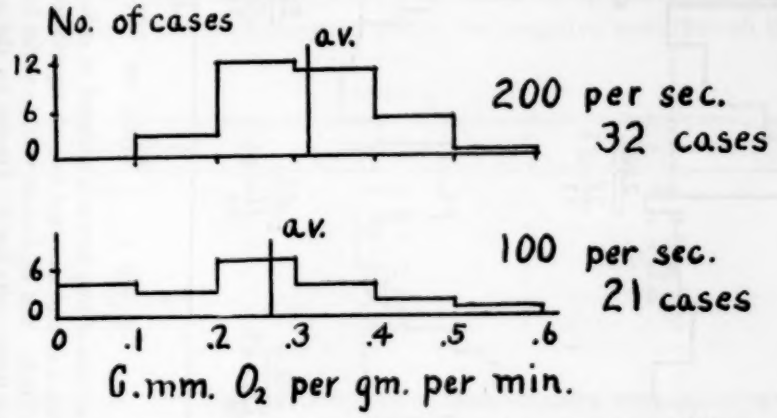

FIG. 2. Frequency diagrams to show the distribution of the values obtained for the excess oxygen consumption due to stimulation at 200 and at 100 shocks per second. The average values were respectively 0.315 and 0.268 c.mm. per gm. of nerve per minute of stimulation.

stimulation were directly compared, the current through the primary coil being equal for both frequencies. The result of this experiment is plotted in Fig. 3, *a*. In the two comparisons there recorded, doubling the frequency increased the excess oxygen used only 0.23/0.20 = 1.15 and 0.28/0.25 = 1.12 times. For purposes of comparison with these figures, the magnitude of the negative variation in nerves similarly stimulated was recorded with a Leeds-Northrup high sensitivity ballistic galvanometer of 2300 ohms resistance. The deflections obtained on stimulating the nerve with 200 shocks per

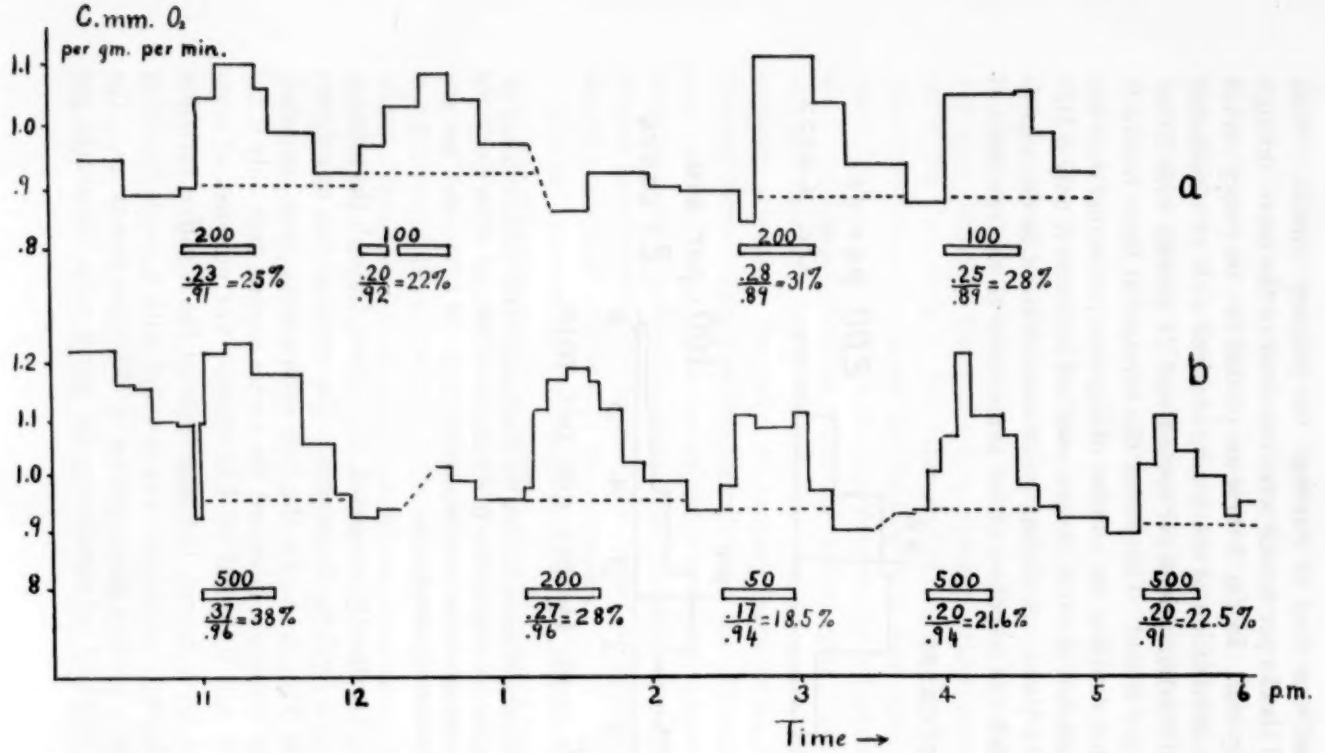

FIG. 3. Graphs showing the rate of oxygen consumption of frog nerves stimulated at different frequencies varying from 50 to 500 shocks per second. The intensity of stimulation was kept constant in each experiment, with the coil set at 13 cm. The voltage drop in the primary was 0.4 volts in *a* and 0.7 in *b*. The equivalent voltage varies in direct proportion to the frequency but for 200 per second it was 0.02 volts for *a* and 0.034 volts for *b*. Figures on the graph show the frequency of stimulation (above) and the percentage increase (below) calculated from the ratio of the excess oxygen per gm. per minute of stimulation and the resting rate per gm. per minute. In *b*, one end of the nerve was crushed which accounts, perhaps, for the high initial rate and led to a great uncertainty in the base line of the first period of stimulation.

second were on the average 1.15 times that found at 100 per second. Through the courtesy of Professor A. V. Hill I am informed that the heat production of nerve is increased 93/67 = 1.4 times for an increase in the frequency of stimulation from 100 to 280 per second or perhaps 1.25 times for an increase in frequency from 100 to 200. The fact that the response of the nerve to this change in frequency, as indicated by its oxygen consumption, is similar to its response as indicated by its negative variation and its heat production, is good evidence that the extra oxygen is actually used to supply energy for the nerve impulse.

In a few preliminary experiments simultaneous measurements have been made of the excess oxygen and of the negative variation on the

TABLE I.

Frequency	Excess oxygen	Negative variation
per sec.	*c. mm.*	*m.v.*
500	0.37	2.12
200	0.27	2.09
50	0.17	1.2
500	0.20	2.16
500	0.20	1.92

same nerve. For this purpose two silver electrodes were introduced into the nerve chamber, one of which was in contact with the injured end of the nerve and the other with its intact surface. The results of one such experiment are plotted in Fig. 3, *b*, and the figures are collected in Table I. The values for the excess oxygen cannot be determined with great precision, but there does seem to be a definite correlation between the excess oxygen and the negative variation over this range of frequencies from 50 to 500 per second. The fact that this tenfold increase in the frequency had so little effect on the oxygen consumption is to be expected from the fact that impulses set up early in the refractory period are subnormal. It may be suspected also that with certain settings of a tuning fork vibrating at 250 per second there would be some interference between the make shock and its rapidly succeeding break shock.

In Fig. 4 there are plotted two frequency curves to show the range

of values obtained for the percentage increase in oxygen consumption from stimulation at 200 shocks per second and for the resting oxygen consumption. The rather wide distribution is perhaps due to the varying conditions of the frogs (*R. pipiens*). For the earlier experiments they were kept in an indoor tank of running water; for the later experiments they were kept in water in a cold room maintained just above the freezing point, and were killed and dissected immediately after removal. No certain differences were noted in the behavior of the nerves in these two cases, however.

My most reliable data were obtained by stimulation at 200 shocks per second, and these may be used for comparison with the heat pro-

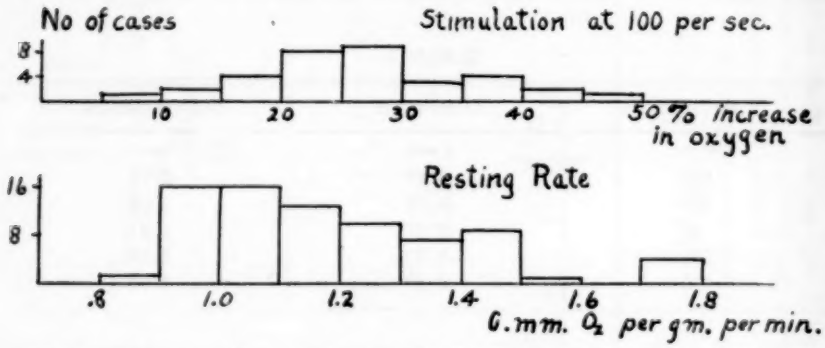

FIG. 4. Frequency diagram showing the distribution of the observed values for the percentage increase in oxygen consumption from stimulation at 200 per second (upper) and for the resting rate of oxygen consumption (lower).

duction of nerve. In 32 periods of such stimulation the resting rate was 1.23 c.mm. of oxygen per gm. of nerve per minute and the excess oxygen used in activity was 0.32 c.mm. of oxygen per gm. of nerve per minute of stimulation, or 26 per cent of the resting rate. The corresponding figures for the dogfish nerve were much the same, *i.e.* 1.35 c.mm. per gm. per minute as a resting rate and an increase on stimulation (100 shocks per second) of 0.21 c.mm. per gm. per minute of stimulation, the percentage increase varying from 10 to 32 per cent. Parker (1925, *b*) has reported a carbon dioxide elimination in the resting frog nerve of 4.46 c.mm. per gm. per minute and an increase due to stimulation of 14 per cent or 0.62 c.mm. per gm. per

minute of stimulation. In absolute magnitude these figures are higher than mine.

Downing, Gerard, and Hill (1926) found a heat production in the frog nerve which was equivalent to an oxygen consumption of 0.75 c.mm. per gm. nerve per minute of stimulation. They stimulated at a frequency of 280 shocks per second. At 100 per second the heat was 67/93 as great (personal communication from Professor Hill), which would have demanded an extra oxygen consumption of 0.54 c.mm. At 200 per second the figure would have been perhaps 0.66 c.mm. per gm. per minute of stimulation. This is about twice as large as the mean value which I have actually observed, *i.e.* 0.32 c.mm., although my highest figures have been over 0.5 c.mm. To account for this discrepancy, it is probable that during the first 10 seconds of stimulation there is a greater energy breakdown than during similar periods at the end of a half hour of stimulation. The heat was measured during the first 10 seconds only.

It is conceivable that even in the small frog nerve in an atmosphere of oxygen the central portion of the proximal end of the nerve, where its diameter is greatest, would be asphyxiated and fail to respond. This would help to explain the discrepancy between the heat production and the oxygen. By making use of Krogh's (1919) diffusion constant for oxygen, however, it can be shown that this is not the case. To do this one proceeds with a cylinder in much the same way that Warburg (1923) has done for the simpler case of a flat disc. Consider a cylinder of nerve of radius a and length l, consuming A cc. of oxygen per gm. per minute. D, the diffusion constant for oxygen in muscle tissues, $= 1.4 \times 10^{-5}$ cc. of oxygen diffusing across a surface area of 1 cm.^{2} per minute under a pressure gradient of 1 atmosphere per cm. The concentration c_0 of oxygen at the surface is kept constant. Diffusion through the ends of the nerve is neglected. In any concentric cylindrical layer, of radii r and $r\text{-}dr$, the oxygen consumption in time dt is

$$A\,[\pi r^2 - \pi\,(r - dr)^2]\,l\,dt = 2\pi\,A\,lr\,dr\,dt \qquad (1)$$

The oxygen diffusing *into* this layer in time dt is

$$D\,2\pi\,rl\,\frac{dc}{dr}\,dt \qquad (2)$$

The oxygen diffusing *out* of this layer is

$$2\pi\, Dl\, (r - dr)\, dt\left(\frac{dc}{dr} - \frac{d^2c}{dr^2}dr\right) \tag{3}$$

Equation (1) = equation (2) − equation (3) or, after simplification,

$$\frac{Dd^2c}{dr^2} + \frac{Ddc}{dr} = Ar \tag{4}$$

The solution of this differential equation is

$$c = c_0 - \frac{A}{4D}(a^2 - r^2), \tag{5}$$

c being the concentration of oxygen in atmospheres at a distance r from the center of the cylinder, c_0 being the concentration at the surface, *i.e.* when $a = r$. Putting $r = 0$ and $a = 0.1$ cm. which is the maximum for frog nerves I have used,

$$c = 1 - \frac{0.00123 \times 0.1^2}{4 \times 1.4 \times 10^{-4}} = 1 - 0.22 = 0.78$$

atmosphere at the center of the nerve. Thus the tension inside the larger end of the nerve is $0.78 \times 760 = 590$ mm. if the nerve is in pure oxygen. If it is in air the tension at the center of the larger end is just reduced to zero.

From (5) it is evident that when in pure O_2 the tension at the center will just reach zero if

$$\frac{A}{4D}a^2 = 1 \text{ or if } a = 0.213 \text{ cm.}$$

which is about the maximum radius of the largest dogfish nerves. The assumption is made that A is independent of the tension of oxygen. This confirms the estimate previously made (Fenn, 1927) that the rate of diffusion of oxygen was a limiting factor in dogfish nerves in air.

I am indebted to an anonymous mathematical colleague for assistance with this equation.

SUMMARY.

1. The resting rate of oxygen consumption of the excised sciatic nerve of the frog is 1.23 c.mm. of oxygen per gm. of nerve per minute.

2. During stimulation with an induction coil with 100 make and 100 break shocks per second there is an excess oxygen consumption amounting on the average to 0.32 c.mm. of oxygen per gm. of nerve per minute of stimulation, or a 26 per cent increase over the resting rate.

3. The magnitude of the excess oxygen consumption in stimulation, in agreement with the all-or-none law, is not markedly influenced by considerable variations in the intensity of stimulation.

4. Increasing the frequency of stimulation from 100 to 200 shocks per second increases the extra oxygen used only 1.12–1.18 times. The same change in frequency of stimulation increases the negative variation 1.15 times and the heat production about 1.25 times (Hill).

5. This parallelism between the excess oxygen and the negative variation argues definitely for some causal connection between the excess oxygen and the nerve impulse itself.

6. Calculation shows that the oxygen tension inside these nerves was not zero.

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THE KINETICS OF DARK ADAPTATION.

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I.

Nature of Contents.

1. Purpose.—Many photosensitive animals acquire in the dark an increased sensibility to light. A quantitative description of this process of dark adaptation is limited, however, to very few animals. For vertebrates, there are the numerous and accurate measurements with man (Piper, 1903; Nagel, 1911; Hecht, 1921–22); the few observations with the chick (Honigmann, 1921); and the measurements with the tadpole (Obreshkove, 1921); while for invertebrates there are only the data with the clam, *Mya arenaria* (Hecht, 1918–19 *b*). Additions to this meager collection of data are obviously desirable. The first purpose of the present paper is to present measurements of the dark adaptation of two additional invertebrates: the lamellibranch, *Pholas dactylus;* and the ascidian, *Ciona intestinalis.*

The original study of the dark adaptation of *Mya* (Hecht, 1918–19 *b*) contained an analysis of the process in terms of a photosensory mechanism. Since then, much has been learned about the nature of the processes involved, and as a result certain modifications in the details of the theoretical treatment have become necessary (Hecht, 1922–23). The second purpose of this paper is to present an analysis of the data of dark adaptation which is in keeping with present knowledge.

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† The experiments here recorded were made at the Zoological Station, Naples, during my tenure of the Jacques Loeb Memorial Table. I wish to express my appreciation of the many kindnesses shown me during my stay in Naples by the Director of the Zoological Station, Dr. Reinhard Dohrn.

The organisms whose dark adaptation has been measured may be divided into two groups depending on the nature of their photosensitive structure, and on the method of making the measurements. In man and the chick the light sensitive system is a complicated vertebrate eye. Dark adaptation in these animals has been measured by finding the illumination intensity just perceptible to the eye during different moments of the stay in the dark. Thus of the two factors which control the photochemical effect of a given light, the time of exposure is held constant while the intensity is varied. However, in the other animals mentioned the photoreceptors are superficially located and are diffuse. In *Mya* and *Pholas* they are on the siphons and on the exposed parts of the mantle (Dubois, 1892; Wenrich, 1916); in *Ciona* they are in a small area between the siphons (Hecht, 1918–19 *a*); and in the tadpole they are in the skin (Obreshkove, 1921). With these animals dark adaptation is measured by finding the exposure required to elicit a response to a given illumination. Time is thus the variable while the intensity is constant.

The principles underlying the theoretical analysis of dark adaptation is the same in these two groups of animals (Hecht, 1919–20). But because of the difference in the method of securing the data for the two classes it is more convenient to present them separately. I shall therefore consider here only the second group. The third purpose of the present paper is to complete the analysis of this class of animals by including a study of Obreshkove's data of the dark adaptation of the tadpole.

It might appear desirable, in order to render this study complete, to collect here the bits of information about dark adaptation which are scattered in the voluminous literature of the sensitivity of animals to light. However, such a course would not be profitable, because this material consists mostly of the mere statement that a given animal can become dark adapted, coupled only occasionally with a numerical datum giving the order of magnitude of this effect (*e.g.* Hess, 1910). An apparent exception might seem to be the work of Folger (1924–25) who, under the heading of dark adaptation records a few measurements of the recovery of *Ameba* from stimulation by light. Folger has very clearly shown, however, that most of this recovery can occur in the light as well as in the dark. The process is, therefore, not dark

adaptation; and is not relevant to the present study dealing with the quantitative aspects of this process.

2. Definitions.—Folger's paper calls for comment here, because of the unexpected meaning which he has attached to the terms dark adaptation and light adaptation.

> "An organism is said to be dark adapted when it will respond to a sudden increase in the intensity of illumination. The converse of dark adaptation is light adaptation. This is brought about by exposure to light. An animal is said to be light adapted when from a lack of dark adaptation it fails to respond to a sudden increase in illumination."[1]

According to Folger's definition of dark adaptation, the human eye, or *Mya*, or *Ciona* are dark adapted in broad daylight, since they all respond to a sudden increase in illumination under such conditions. Similarly according to Folger, none of these organisms can become light adapted, since there are no light conditions known under which they do not respond to a sudden increase in illumination if of sufficient magnitude. It is therefore to be regretted that an erroneous and confused connotation has been applied to such terms as dark and light adaptation which have always had a precise and accepted meaning.[2]

Aubert (1865) introduced the term adaptation into physiology when he recorded the first measurements of the dark adaptation of the eye. Since then there has been no misunderstanding of its meaning. A detailed treatment of it has been given by Nagel (1911). This is too long to be quoted here; I shall therefore summarize it briefly in a form applicable to the present situation. A dark adapted animal is one whose sensitivity to light has reached a constant value as the result of a sustained stay in the dark. A light adapted animal is one whose sensitivity to light has reached a constant value as the

[1] Folger (1924–25, p. 279).

[2] This confusion is only one of the many to be found in Folger's paper,—a situation which may be best illustrated perhaps by quoting one of the major conclusions of the work (p. 290). Folger is concerned with the fact that "*A mechanical shock exerts a distinct effect upon the reaction to light.*" "*This indicates that increase in illumination and mechanical shock produce the same changes in Amoeba, that mechanical stimulation and photic stimulation are fundamentally the same. If this is true, it is evident, since mechanical stimulation is not photochemical, that photic stimulation cannot be photochemical.*"

result of a sustained exposure to a given intensity of illumination. The process of reaching these two states is that of dark adaptation and of light adaptation, respectively. These definitions might be supposed to be self-evident. The terms which they describe will therefore be used in their accepted sense in the present paper.

II.

Experiments with Pholas dactylus.

1. General Properties of Sensibility.—Pholas is an animal whose photosensory behavior is similar to that of *Ciona* and *Mya*. Its resemblance to *Mya* in particular is so extraordinary that in a short time it is possible to demonstrate in a qualitative way the existence of all the interrelations which have been quantitatively established in *Mya* (Hecht, 1925). The following are some of these properties; they are recorded here so that the study of the dark adaptation of *Pholas* may be intelligible.

(*a*) *Pholas* is very sensitive to light, and responds to it by a vigorous retraction of its extended siphon. The time from the beginning of the exposure to the beginning of the retraction was called the "latent time" by Dubois (1892) who first worked with this animal. In conformity with the less accurate but more usual practice it will be referred to as the "reaction time."

(*b*) This reaction time is not a simple period. As in the case of *Ciona* and *Mya*, it is composed of two parts, an exposure period and a latent period. The first is occupied by the necessary exposure to light. It varies with the intensity and can be very short with high intensities. The second, or latent period, occupies most of the reaction time. During the latent period the animal may remain in the dark and still respond after the usual reaction time. Thus a reaction time of 2 seconds to an illumination of 500 meter candles is composed almost entirely of latent period, because the actual exposure to light need be only 0.02 second.

(*c*) Up to a certain limit of exposure the latent period varies inversely with the duration of the exposure.

(*d*) Temperature has almost no influence on the exposure period, as is to be expected if the exposure is concerned with a photochemical

reaction. The latent period, however, is definitely influenced by temperature, in a manner similar to most "dark" reactions.

(*e*) *Pholas* comes into sensory equilibrium with any illumination to which it is exposed continuously. Its first act on being illuminated is to retract its siphon. In a few seconds, however, it extends the siphon and appears to be unstimulated by the light. Its sensitivity is now much less than before, because the intensity of illumination has to be considerably augmented in order to cause *Pholas* to respond again.

(*f*) Its original sensibility may be restored by placing the animal in the dark. This is a fairly slow process in *Pholas*, as will be apparent presently.

Dubois (1892) failed to note many of these properties of the sensory process, because of his interest in the contraction of the siphon. He laid great stress on the fact that the siphon response may be recorded graphically, and most of his work was concerned with the form of the siphon contraction under different conditions of stimulation. To Dubois is due the important point that the photosensory process and the resulting retraction response are essentially independent of the rest of the animal, because he showed that a detached siphon retains its sensibility to light and its capacity to contract for several days after removal from the rest of the animal.

2. Dark Adaptation.—If after exposure to strong light, *Pholas* is placed in the dark, its reaction time to a light of constant intensity decreases steadily until it reaches a constant minimum characteristic of its response to that intensity. The course of this dark adaptation has been investigated in the following manner. An animal is exposed to an illumination of 10,000 meter candles. After the response, it almost at once expands its siphon. 2 or 3 minutes seem to be sufficient for light adaptation, but 7 minutes are allowed in these experiments. The animal is then placed in the dark and during the next 2 hours its reaction time to an illumination of 30 meter candles is measured three times. Several hours later the animal is again light adapted as before; it is placed in the dark and its reaction time to 30 meter candles is measured at such times that all together there are secured six values of the reaction time at half hour intervals during dark adaptation. The measurements are made in two series instead of one, so as to

allow an hour between successive exposures. In this way the progress of dark adaptation is disturbed as little as possible by the short exposures to light needed for making the measurement.

During the stay in the dark the animal in its rectangular glass dish is kept in a water bath so as to maintain the temperature constant. The source of light is properly screened in a box, and water filters are interposed to reduce the heat to a minimum. Though negligible during a measurement, this factor must be controlled during the 7 minutes light adaptation when the temperature is kept constant by the addition of cold sea water to the dish.

TABLE I.

Dark Adaptation of Pholas. 18 Animals. Temperature, 16.5°C. Latent Period, 1.00 Second. $k = 0.0143$; $a = 0.730$.

Time in dark t	Reaction time r	
	Observed	$r = \frac{95.8 + t}{25.9 + t}$
min.	*sec.*	*sec.*
10	2.95	2.95
30	2.23	2.25
60	1.81	1.81
90	1.65	1.60
120	1.46	1.48
150	1.40	1.40

With *Pholas* I measured the dark adaptation of 18 animals. The data secured are given in Table I, and graphically in Fig. 1. It is apparent that the process is regular, and at this temperature takes more than 3 hours to become complete.

3. Theoretical Analysis of Dark Adaptation.—The reaction time of *Pholas* to light is an interval occupied by several processes which combine to produce a constant effect, namely a siphon retraction. The algebraic sum of the velocities of these separate processes is represented by the reciprocal of the reaction time. Only two of these processes, the photochemical and the latent period, need be considered, since the time occupied by conduction and central nervous activity is negligible in stop-watch measurements and may be included in the latent period. The data therefore show that the sum of the velocities of the two major processes increases during dark adaptation.

The work with *Mya* (Hecht, 1922–23; 1923–24) has shown that to cause a given photosensory response a definite and constant amount of photochemical decomposition must be produced by the incident light. Let us assume that this holds during the dark adaptation of *Pholas*, and therefore that the photochemical effect during each re-

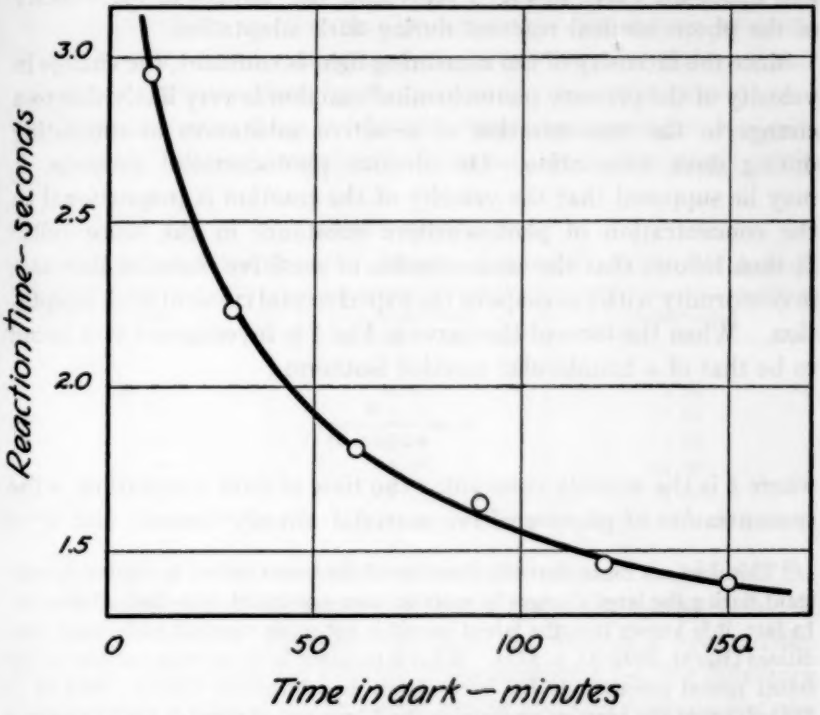

FIG. 1. Dark adaptation of *Pholas*. Each point is the average of 18 measurements, one with each of 18 animals. The first, third, and fifth points on the curve for each animal were made during one run of dark adaptation, while the other three points were made during another run several hours later. The curve is calculated in terms of a bimolecular reaction.

action time in Table I is constant. This is the only assumption which has to be made in order to understand dark adaptation.

It is already well known that the velocity of the latent period is directly proportional to the magnitude of photochemical effect produced during the exposure (Hecht, 1918–19 *c*; 1925–27). If the photo-

chemical effect be assumed constant, it follows that the velocity of the latent period is also constant.[3] The total velocity of the reaction time processes is thus composed of two velocities, one of which is constant. Any increase in total velocity must therefore represent an increase in the velocity of the primary photochemical process only; and hence the curve in Fig. 1 represents the changes in the velocity of the photochemical reaction during dark adaptation.

Since the intensity of the measuring light is constant, the change in velocity of the primary photochemical reaction is very likely due to a change in the concentration of sensitive substances accumulating during dark adaptation. On obvious photochemical grounds, it may be supposed that the velocity of the reaction is proportional to the concentration of photosensitive substance in the sense cells.[4] It then follows that the concentration of sensitive material increases in conformity with the shape of the experimental curve of dark adaptation. When the form of the curve in Fig. 1 is investigated it is found to be that of a bimolecular reaction isotherm

$$k_1 = \frac{x}{t\,a\,(a-x)} \tag{1}$$

where k is the velocity constant; t the time of dark adaptation; x the concentration of photosensitive material already formed; and $a-x$

[3] This does not mean that the duration of the latent period is necessarily constant during the large changes in reaction time associated with dark adaptation. In fact, it is known that the latent period is not quite constant under such conditions (Hecht, 1922–23, p. 573). What is constant is the average velocity of the latent period process. It has been shown experimentally (Hecht, 1918–19 *c*; 1925–27) that the reaction underlying the latent period depends for its progress on the products formed by the photochemical reaction during the exposure. If the exposure is long, the latent period reaction begins as soon as some photochemical products are formed, and increases in velocity as these increase in concentration. The average velocity of the latent period process is then constant, though its actual duration may vary slightly. If, however, the necessary exposure is short in comparison with the latent period the former may be considered instantaneous, and therefore both the velocity of the latent period and its duration will be constant.

[4] This proportionality between concentration of sensitive material and velocity of reaction is a first approximation, and holds strictly in an irreversible reaction only. However, since the analysis in terms of it is descriptive of the data, it is retained in its simple form without the added term for the reversible reaction.

the concentration still to be formed. The third column in Table I gives the calculated values of the reaction time on which the theoretical curve in Fig. 1 is based.

It is necessary to describe in detail the method of calculating the terms in the above equation in order to show how they are derived from the experimental data of Table I. The limits between which the velocity of the reaction time processes can vary are the minimum velocity at the first moment of dark adaptation, and the maximum possible velocity as given by the velocity of the latent period alone. The range between these two limits represents the possible variation in the speed of the photochemical reaction, and corresponds to the

TABLE II.

Determination of Latent Period of Pholas. Temperature, 16.5°C. 16 Animals.

Exposure	Reaction time	Latent period
sec.	*sec.*	*sec.*
0.11	1.47	1.36
0.20	1.37	1.17
0.28	1.28	1.00
0.35	1.27	(1.00)
0.41	1.28	(1.00)

total change a in concentration of sensitive material S. If x is the concentration of S at the moment t, then $a - x$ is the concentration of S still to be formed, and corresponds to the difference between the maximum velocity and the velocity at the moment t; *i.e.*, the difference between the reciprocal of the latent period and the reciprocal of the reaction time at the moment t.

The reaction time values are of course the measurements in Table I. The latent period is determined by a separate experiment with the same animals. An animal is stimulated at hourly intervals by a series of graded exposures, and the reaction time measured. Table II gives the data for 16 of the animals used in Table I. It is apparent that the smallest exposure giving the minimum reaction time of 1.28 seconds is 0.28 second. The minimum reaction time minus the minimum exposure is obviously the latent period, and is equal to 1.00 second.

The value $a - x$ at the moment t is thus a difference between the reciprocals of two experimentally determined magnitudes. If now $\frac{1}{a - x}$ is plotted against t the points should lie on a straight line

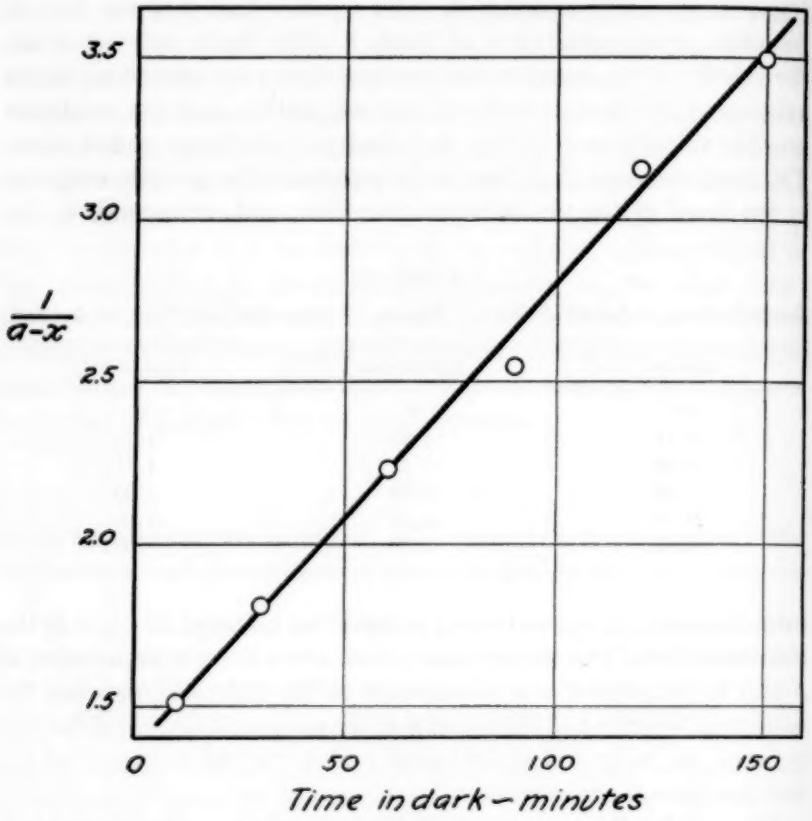

FIG. 2. Dark adaptation of *Pholas*. Same data as in Fig. 1. The ordinates are $r\,p\,/\,(r - p)$, which is equivalent to $1\,/\,(a - x)$ in the equation for a bimolecular process. Here r is the reaction time and p the latent period.

provided the reaction is bimolecular. This is because equation (1) can be converted into the form of a straight line

$$\frac{1}{a - x} = kt + \frac{1}{a}. \tag{2}$$

If we call r the duration of the reaction time, and p that of the latent period, then $a - x$ will be represented by $1/p - 1/r$, and $\frac{1}{a - x}$ by $\frac{rp}{r - p}$ according to the above reasoning. Fig. 2 shows that if $\frac{1}{a - x}$ or its equivalent $\frac{rp}{r - p}$ is plotted in this way, the data conform to equation (2). The tangent of the line is k, the reciprocal of its intercept on the ordinate axis is a. The significance of this graphic mode of computation is that the values of k and a are both found directly from the experimental data, and are not arbitrarily assumed in the calculations.

4. Interpretation.—In making the above analysis no assumption was made with regard to the way in which the sensitive material accumulates in the sense cells during dark adaptation. The agreement between the kinetics of a simple bimolecular reaction and the course of dark adaptation permits one to draw the conclusion that the process is chemical in nature. If the kinetics of dark adaptation had followed a monomolecular isotherm this interpretation would have been doubtful because under certain conditions the course of a diffusion process may also be described that way. As it is one must suppose that two substances at least are concerned in the formation of the sensitive material, and that these two substances combine in a chemical manner.

It is apparent that the chemical nature of the process can be corroborated by studying the effect of temperature on dark adaptation. This was not done with *Pholas*, but has been done with *Mya*, and the results as given in the next section bear out the chemical interpretation of the present data.

III.

Mya arenaria.

1. Assumptions.—The original study with *Mya* contained an analysis of its dark adaptation on the basis of two assumptions. These were first, that the photochemical effect of light on the sensory system is directly proportional to the exposure; and second, that the concentration of photochemical decomposition products required for a

response is proportional to the concentration of products already present in the sense cell. In terms of them dark adaptation was shown to follow the kinetics of a bimolecular reaction.

However, both assumptions have proven inadequate for the further handling of the characteristics of the photosensory process, and have been discarded (Hecht, 1922–23). Therefore, it is proposed to show here how from the same data the same conclusion may be reached by the simpler and more plausible analysis just used with *Pholas*. This entails only one assumption: under all circumstances a definite amount of photochemical decomposition is required to produce a

TABLE III.

Dark Adaptation of Mya. Data from Hecht. 6 Animals. Temperature, 22.0°C.*

Time in dark	Reaction time		
	Observed	Calculated in original way	Calculated in present way
min.	*sec.*	*sec.*	*sec.*
4	2.28	2.21	2.24
8	1.95	1.92	1.94
14	1.70	1.71	1.74
20	1.60	1.60	1.63
29	1.51	1.53	1.55
40	1.50	1.48	1.49
53	1.50	1.47	1.45

*Hecht (1918–19 *b*), p. 551.

given sensory effect. Though given here as an assumption purely, its validity is attested by a good deal of experimental evidence (Hecht, 1922–23; 1923–24).

2. Dark Adaptation.—The data, given in Table III, are the original ones for *Mya*, and were secured by methods which are essentially those described above for *Pholas*. With the data are the values calculated in the present manner as given in the previous section with *Pholas*, and in the old manner. It is clear that the two modes of theoretical treatment give the same approximation to the experimental data. This follows from the fact first, that the curve of dark adaptation is an hyperbola, and second, that the two methods of

calculation involve the use either of a given value of the ordinates (reaction time) or of its reciprocal.

It may not be amiss to indicate precisely why the two methods give almost identical results. Let r_t be the reaction time after t minutes of dark adaptation, and t_o its value at the first moment when $t = 0$. Let p be the latent period. Then according to the original hypothesis

$$a = k_2 (r_o - p) \tag{3}$$

$$x = k_2 (r_o - r_t) \tag{4}$$

$$a - x = k_2 (r_t - p) \tag{5}$$

where a is the total amount of sensitive material to be formed, x the amount already formed, and $a - x$ the amount still to be formed from the precursors according to the usual equation

$$k_1 = \frac{x}{t\, a\, (a - x)} \tag{1}$$

for a bimolecular process. If the corresponding values in (3), (4), and (5) are substitued in (1) it becomes

$$k = \frac{r_o - r_t}{t\, (r_o - p)\, (r_t - p)} \tag{6}$$

which describes the behavior of the experimental data. According to the present alternative hypothesis it is

$$a = k_3 \left(\frac{1}{p} - \frac{1}{r_o} \right) \tag{7}$$

$$x = k_3 \left(\frac{1}{r_t} - \frac{1}{r_o} \right) \tag{8}$$

$$a - x = k_3 \left(\frac{1}{p} - \frac{1}{r_t} \right) \tag{9}$$

which describe the relations, and which give

$$k = \frac{\dfrac{1}{r_t} - \dfrac{1}{r_o}}{t \left(\dfrac{1}{p} - \dfrac{1}{r_o} \right) \left(\dfrac{1}{p} - \dfrac{1}{r_t} \right)} \tag{10}$$

when substituted in equation (1). Equation (10) reduces very simply to

$$\frac{k}{p^2} = \frac{r_o - r_t}{t\,(r_o - p)\,(r_t - p)} \tag{11}$$

which, since p is constant, is identical in form with equation (6).

The choice between the two hypotheses is therefore not so much as to which gives a better agreement with the data of dark adaptation, but as to which is more consistent with other work, and inherently perhaps more plausible.

TABLE IV.

Dark Adaptation of Mya at Different Temperatures. Data from Hecht. 5 Animals.*

11.5°C.; $k = 0.0744$			16.2°C.; $k = 0.124$			21.9°C.; $k = 0.217$		
Time in dark	Reaction time		Time in dark	Reaction time		Time in dark	Reaction time	
	Observed	Calculated		Observed	Calculated		Observed	Calculated
min.	*sec.*	*sec.*	*min.*	*sec.*	*sec.*	*min.*	*sec.*	*sec.*
11.	5.04	4.72	4.	3.50	3.56	4.	2.30	2.25
15.	4.13	4.31	8.	2.98	2.97	8.	1.98	1.95
20.	3.94	3.94	13.5	2.44	2.56	13.	1.70	1.76
29.5	3.50	3.48	20.	2.30	2.31	19.5	1.64	1.64
39.5	3.11	3.20	30.	2.12	2.10	29.	1.54	1.55
55.	2.92	2.92	40.5	2.05	1.98	39.5	1.52	1.49
			54.	1.90	1.89	54.5	1.45	1.45

* Hecht (1918–19 *b*), p. 556.

3. Dark Adaptation and Temperature.—The present theoretical analysis of the data of *Mya* brings to light a quantitative relation between dark adaptation and temperature. The purpose of the original experiments with temperature was to measure the temperature coefficient of dark adaptation in order to ascertain whether the process underlying dark adaptation is chemical in nature, in this way corroborating the evidence from its kinetics. The temperature coefficient, Q_{10}, for the velocity constant k confirmed this by being near 2.5. Nevertheless the values of k as derived from the original computations did not fit the Arrhenius equation relating temperature with the velocity constant of a chemical reaction (Arrhenius, 1912).

It is a significant point that recalculation of the same data in terms of the present analysis yields a series of values for the velocity constant k which show an excellent agreement with the Arrhenius equation.

The experimental data for dark adaptation at three temperatures are given in Table IV. They are shown graphically in Fig. 3 accord-

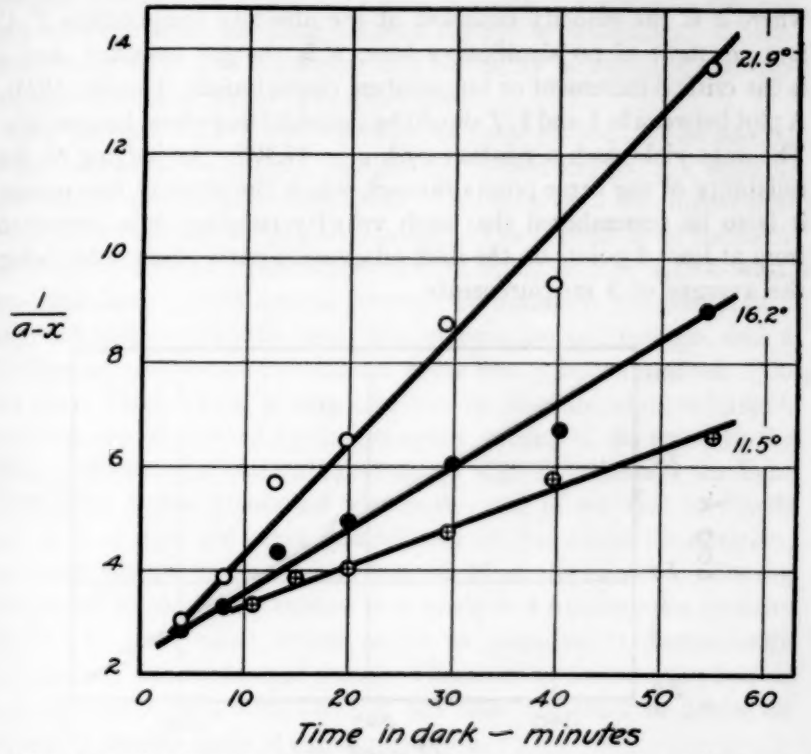

FIG. 3. Dark adaptation of *Mya* at three temperatures. The ordinates are $rp / (r - p)$ which is equivalent to $1 / (a - x)$ in the equation for a bimolecular process. The velocity of the reaction as indicated by the slope of the lines increases with the temperature.

ing to the method of analysis described above. The corresponding calculated values are also given in Table IV, from which, as well as from Fig. 3, may be noted the adequacy of a bimolecular reaction for describing dark adaptation.

The velocity constants k derived from these data are given in

Table IV, and their relation to the temperature in Fig. 4. For purposes of graphic presentation the Arrhenius equation may be written

$$ln\, k = -\frac{\mu}{RT} + C \tag{12}$$

where k is the velocity constant at the absolute temperature T, C is a constant of no significance here, R is the gas constant, and μ is the critical increment or temperature characteristic (Crozier, 1924). A plot between $ln\, k$ and $1/T$ should be a straight line whose tangent is μ. The data yield such a relation with $\mu = 17{,}400$. In judging of the reliability of the three points through which the straight line passes, it is to be remembered that each velocity constant k is computed from at least 6 points on the dark adaptation curve, each point being the average of 5 measurements.

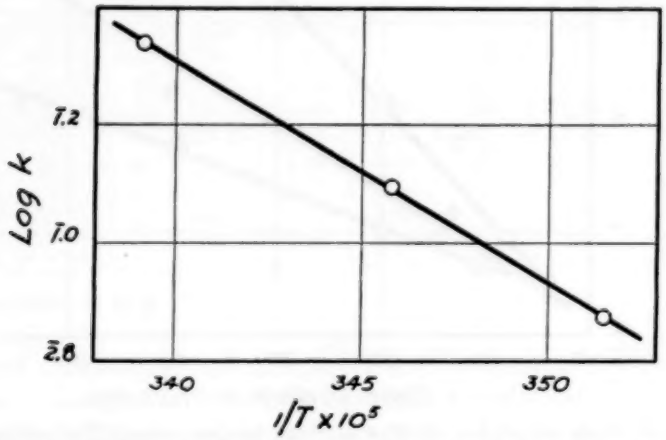

FIG. 4. Relation between temperature and the velocity constant k of the dark adaptation reaction. The straight line represents the Arrhenius equation.

The effect of temperature on dark adaptation in *Mya*, therefore, not only shows the process to be bimolecular at all temperatures investigated, but demonstrates as well that the velocity constant of the process bears a well known theoretical relation to the temperature such as is usually found in chemical reactions.

IV.

Ciona intestinalis.

1. Method.—In presenting the data obtained with *Pholas* and *Mya* only the barest manipulative essentials have been mentioned. Both species, though very sensitive to light, are hardy laboratory animals and tolerate much handling without change of sensibility. Therefore the methods used are simple and can easily be acquired anew by any one wishing to repeat the experiments.

The problem with *Ciona*, however, is more difficult. The species is not very sensitive to light, and in order to measure its dark adaptation a high illumination is required. This involves a still higher illumination for the initial light adaptation. The animals must therefore be brought close to a powerful source of light, such as a 1000 watt, gas-filled lamp of 2000 candle power. To maintain the animal at a constant temperature the usual heat screens are not enough, and a continuous addition of ice-cold sea water has to be maintained. On the other hand, *Ciona* is very sensitive to mechanical disturbances, and is easily stimulated by the necessary stirring of the water in the dish. Moreover, a sudden exposure to high illumination for light adaptation makes it contract vigorously; and in contrast to *Pholas* and *Mya* it does not relax immediately on continued illumination, and may remain contracted as long as 15 or 20 minutes. During this period its own water current is so small that stirring must be more continuous than usual, which serves to stimulate it mechanically and prolong the contracted state. Therefore to secure reproducible and significant data with *Ciona* it has been necessary to devise an especially careful mode of experimentation.

About 25 animals are isolated, each in a separate rectangular dish 5 cm. wide, 10 cm. long, and 7 cm. high, and kept in the dark overnight. Next morning their reaction time to a standard illumination is measured; as a result of which it is possible to select 5 or 6 animals which give a vigorous and clean-cut response to illumination, and which do so in about the same reaction time. Each animal so selected is then carried through the following steps.

It is light adapted. The intensity of the lamp is reduced by means of a rheostat until the filament barely glows. *Ciona* may be brought

as close as 30 cm. to such a light without responding. During a period of half an hour the current is increased up to 200 volts so gradually that the animal does not at any time contract to light, and at the end of the period it is illuminated with 10,000 meter candles. The temperature is kept constant by the very careful addition of small amounts of ice-cold sea water which is distributed by the vigorous current of the fully expanded animal. The animal remains at this high illumination for 6 minutes. At a given moment the illumination is discontinued sharply by means of a shutter, and dark adaptation begins. The manipulations from now on are carried on by the light of a dim ruby lamp to which *Ciona* is not sensitive.

The animal in its dish is placed in complete darkness in a thermostat and kept at a constant temperature for the next 7 or 8 hours. At certain times during dark adaptation the dish is carefully taken out and placed near the lamp in such a position that the animal will receive on exposure an illumination of 6000 meter candles. A minute is allowed to elapse; the animal is exposed by means of a shutter; its reaction time is recorded with a stop-watch; the light is turned off immediately; and the animal is returned to the thermostat. The exact moments when these measurements are made vary somewhat in the different series, but are the same for all the animals in a series. The first measurement is usually made 15 or 20 minutes after the beginning of dark adaptation; the remaining ones at hourly intervals thereafter. If it is desired to have readings closer together the entire procedure of light and dark adaptation is repeated the next day, and readings are made at intervals between those taken the day before.

2. Data.—After many preliminary experiments involving the elimination of errors and development of technic, I measured the dark adaptation of 61 individuals grouped in ten series of experiments. To illustrate the type of result secured with *Ciona* the detailed data of one series of experiments are given in Fig. 5. It is to be noted about Fig. 5 that (*a*) the points given are individual measurements of the reaction time, not averages; (*b*) each animal was measured on 2 successive days, three readings being made each day; (*c*) the curve drawn through the points for each animal is a theoretical one derived as will presently be described. This series is typical; five or six similar ones could be given.

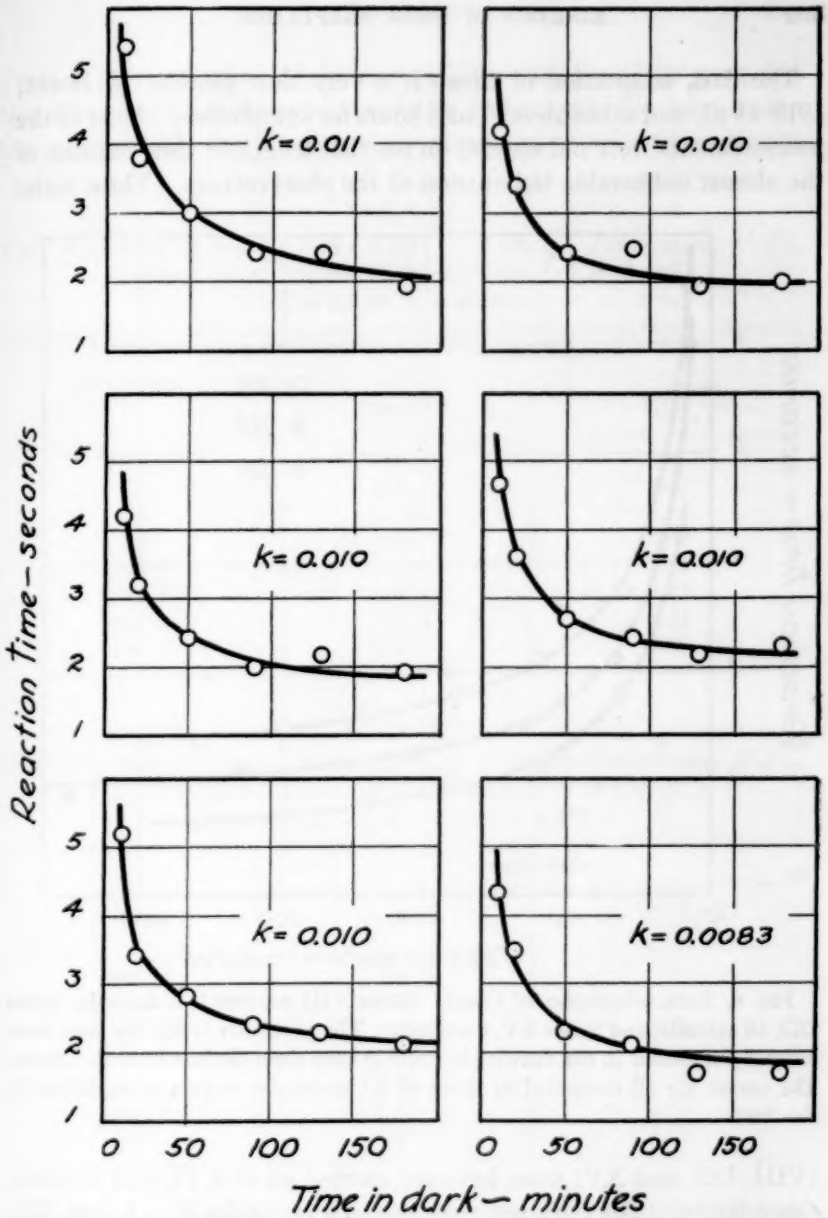

FIG. 5. Dark adaptation of *Ciona*. Each point is a single reading of the reaction time of a single animal. With each animal the points were secured in two runs of dark adaptation; the first, third, and fifth were made on 1 day, the remaining three on the following day. The curve in each case is calculated from the equation of a bimolecular reaction.

The dark adaptation of *Ciona* is a very slow process (*cf.* Hecht, 1918–19 *a*), and takes about 7 or 8 hours for completion. Most of the measurements were not carried on for that length of time because of the almost unbearable tediousness of the observations. Three series

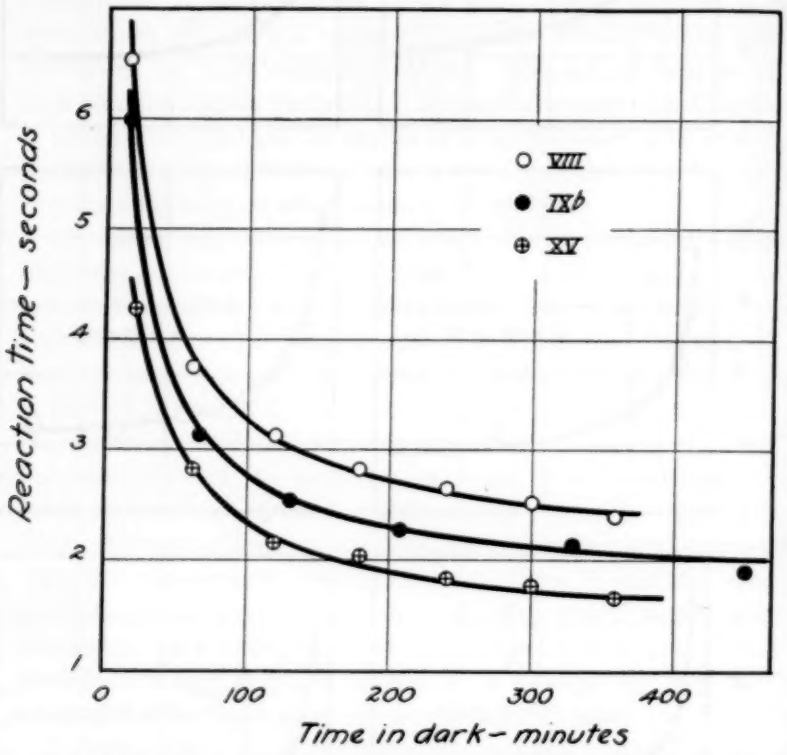

FIG. 6. Dark adaptation of *Ciona*. Series VIII represents 5 animals; Series IX*b*, 10 animals; and Series XV, 6 animals. The points for Series XV have been lowered 0.5 second in the drawing in order to keep them distinct from the others. The curves are all computed in terms of a bimolecular rection as explained in the text.

(VIII, IX*b*, and XV) were, however, carried on to 6, 7½, and 6 hours, respectively. The data secured are given in Tables V, VI, and VII, and in Fig. 6.

3. Interpretation.—The mathematical treatment of the data of

Ciona is the same as of *Pholas* and of *Mya*, with the slight exception that the upper limit of the total velocity, instead of being taken as the latent period velocity, is taken as the maximum reaction time velocity

TABLE V.

Dark Adaptation of Ciona. Series VIII. 5 Animals. Temperature, 15.8°C. Reaction Time at Complete Dark Adaptation Is 2.10 Seconds. $k = 0.0336$; $a = 0.385$.

Time in dark t	Reaction time r	
	Observed	$r = \frac{162.3 + 2.1\,t}{14.7 + t}$
min.	*sec.*	*sec.*
15	6.54	6.53
60	3.74	3.86
120	3.14	3.08
180	2.84	2.78
240	2.66	2.62
300	2.52	2.52
360	2.39	2.45

TABLE VI.

Dark Adaptation of Ciona. Series IXb. 10 Animals. Temperature, 17.1°C. Reaction Time at Complete Dark Adaptation ($t = 16$ Hours) Is 1.82 Seconds. $k = 0.0324$; $a = 0.472$.

Time in dark t	Reaction time r	
	Observed	$r = \frac{119.2 + 1.82\,t}{9.30 + t}$
min.	*sec.*	*sec.*
15	5.99	6.03
65	3.15	3.20
130	2.54	2.55
210	2.29	2.29
330	2.14	2.12
450	1.87	2.04

obtainable at complete dark adaptation. This, like the latent period, is also an experimentally determined value.

The data as with *Pholas* and *Mya* fall on the familiar hyperbola

corresponding to a bimolecular reaction. The curves in Fig. 6 are all calculated in this way, and they show, as do the comparisons in Tables V, VI, and VII, that the agreement between calculation and experiment is very good.

TABLE VII.

Dark Adaptation of Ciona. Series XV. 6 Animals. Temperature, 16.5°C. Reaction Time at Complete Dark Adaptation Is 1.82 Seconds. $k = 0.0268$; $a = 0.426$.

Time in dark	Reaction time r	
	Observed	$r = \frac{159.0 + 1.82\,t}{19.7 + t}$
min.	*sec.*	*sec.*
22	4.77	4.77
60	3.35	3.36
120	2.67	2.70
180	2.53	2.44
240	2.31	2.29
300	2.24	2.21
360	2.13	2.14

TABLE VIII.

Dark Adaptation of Frog Tadpole. Data from Obreshkove. 4 Animals. Latent Period, 1.0 Second. $k = 0.00205$.*

Time in dark t	Reaction time	
	Observed	Calculated
min.	*sec.*	*sec.*
10	52.3	52.3
20	30.0	28.7
30	20.6	20.6
40	15.2	15.3
50	12.8	12.8
60	12.7	10.8

* Obreshkove (1921), p. 268.

V.

Tadpoles.

1. Data.—In the course of some experiments on the photic responses of frog tadpoles, Obreshkove (1921) measured the dark adaptation

of 4 animals. The results are so regular that they must surely be of theoretical interest. Obreshkove, however, merely presents the data without entering into a study of their meaning.

The tadpoles, when exposed to illumination, execute a specific reaction in the nature of a sudden forward movement after a definite reaction time. This response, curiously, is not mediated through the

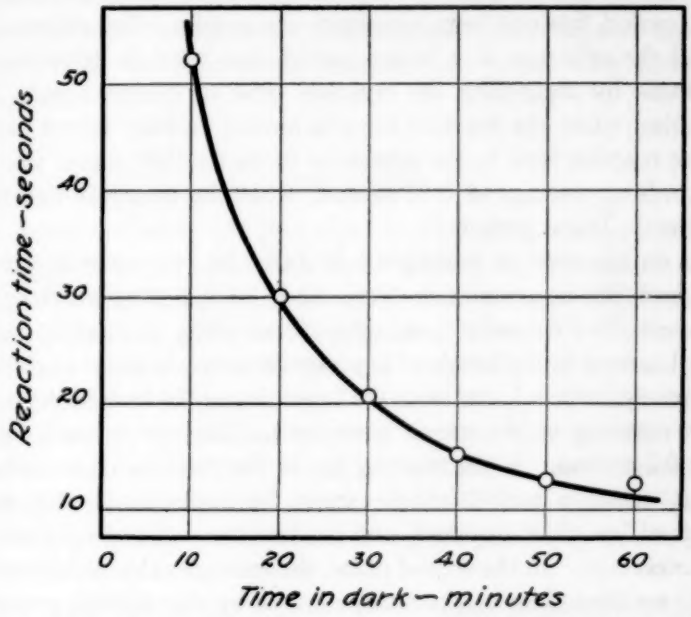

FIG. 7. Dark adaptation of frog tadpoles. Data from Obreshkove (1921). Each point is the average of 4 animals. The curve here again is calculated on the basis that dark adaptation depends on the kinetics of a bimolecular reaction.

eyes, because removal of both eyes does not alter the sensibility of the animal either qualitatively or quantitatively.

The method of measuring dark adaptation employed by Obreshkove is identical with that described for *Ciona*, *Mya*, and *Pholas*. The animal is exposed to an illumination of 10 meter candles for 1 hour, after which it is placed in the dark and at regular intervals its reaction time to the same light of 10 meter candles is measured. The data are given in Table VIII and Fig. 7, in which each point is the average of four readings, one with each of 4 animals.

2. Interpretation.—The theoretical treatment of these data is identical with those of *Pholas* and *Mya*. Dark adaptation conforms to the kinetics of a bimolecular process. The agreement between experimental and calculated values is shown both in Table VIII and in Fig. 7, where the smooth curve is the course of the process computed in the same manner as with the other animals.

The actual handling of the data involves the difficulty that the latent period has not been measured accurately. Obreshkove recognized the existence of a latent period, and tried to determine its magnitude by measuring the reaction time to comparatively high intensities, when the reaction time is almost entirely latent period. For the reaction time to the maximum intensity (500 meter candles) he records an average of 0.76 second, which he treats as the upper limit for the latent period.

This datum must be considered doubtful for two reasons. In the first place the measurements were made with a stop-watch. This instrument is obviously incapable of recording accurately values below 1 second in the hands of any experimenter, because of the time lost from eye to hand, and from the beginning of the finger contraction to the releasing of the watch movement. The eye-to-hand time is about 0.2 second. If the starting lag of the stop-watch is added to this, it makes a period which cannot be neglected in stop-watch records of less than 1 second, and renders the value of such records very uncertain. In the second place, the average value of 0.76 second for the reaction time is apparently derived by the dubious process of not counting the values which are above 1.0 second. Obreshkove gives the 29 individual measurements for this particular intensity. Of them 22 are below 1.0 second; 7 are above. By omitting these 7 one gets 0.76 as an average. The average of all the experimental values, since there seems to be no reason for omitting any, is 0.94 second. Neither of these values is an accurate measure of the latent period. But they indicate that its duration is of the order of 1 second.

Fortunately this is all that is necessary. The values of the reaction time during dark adaptation are quite large: the longest is 52.3 seconds, and the shortest is 12.7 seconds. An error of even 0.5 second in the latent period introduces an error of 1 per cent and 4 per cent,

respectively, in the maximum and minimum exposures. I have therefore assumed the latent period to be 1.0 second and have used this value in computing the figures in Table VIII and the curve in Fig. 7. Actually, if the latent period be assumed as 0.7 second or as 1.3 second it makes no noticeable difference in the computed values as given in this table and figure.

The data of the dark adaptation of the tadpole are therefore represented by the isotherm of a bimolecular reaction, and show the adequacy of the theoretical treatment to which they have been subjected.

VI.

CONCLUSION.

1. Reaction Order.—At first sight it may seem strange that the 3 animals here investigated and the 1 studied by Obreshkove should all show a dark adaptation whose kinetics correspond to the same order of reaction. This is not surprising, however, on intimate acquaintance with the animals, because the similarity of their photosensory make-up is apparent in a variety of ways: in their response to light; in the composition of their reaction time; in the effect of temperature on the two parts of their reaction time; etc. The identity of the order of the reaction underlying dark adaptation is thus only one more property which points to a basic similarity of the photosensory process in these species.

This must not be interpreted to mean that the actual substances entering into the reaction are identical in all the species. In fact, there is evidence—such as the specific spectral sensitivity, and the specific effects of temperature, to be published shortly—which shows that these substances are not the same. It is merely their arrangement and the order of their reaction—in short their organization—which seem to be identical in the different animals.

That the reaction underlying dark adaptation is bimolecular and not monomolecular is not unexpected. Dark adaptation is a process in the course of which a sensitive material accumulates in the sense cell as the result of a chemical reaction. If one supposes that the sensitive material is built up mainly from the products of its photolysis, then a bimolecular reaction is almost the simplest process conceivable.

2. Photosensory Mechanism.—The idea of such a reversible system has been at the basis of all the work which has grown out of the original experiments with *Mya*, and which in turn have served to define that system more rigorously.

It is supposed that a photosensitive material, S, is decomposed by light into at least two substances, P and A, which at the same time tend to recombine and form the original material, S. This reversible reaction has been referred to as the primary photochemical reaction. By itself it is sufficient to account for only a part of the characteristics of the photosensory process in these animals. The existence of a latent period whose duration is dependent on the primary photochemical reaction calls for an additional process in the sense cell. An inactive substance, L, is converted into an active form, T, a definite accumulation of which sets off, electrically or chemically, the nerve-ending attached to the sense cell and thus starts the train of events culminating in the specific response of the organism. This latent period reaction $L \rightarrow T$ can proceed only in the presence of P and A freshly formed from S by the primary photochemical reaction. This relation is probably a simple catalysis.

In a diagrammatic way these two interrelated reactions may be written

$$\xrightarrow{\text{light}} \boxed{\begin{array}{l}\text{light} \\ S \rightleftharpoons P + A;\ L \,\|P + A\,\| \rightarrow T \\ \text{"dark"}\end{array}} \xrightarrow{\text{impulse}}$$

where $\|P + A\|$ means catalysis by $P + A$. The rectangle represents the sense cell. Light comes in at one end and the impulse for the nerve leaves the other end. The two reactions are given here as separated in space; it is to be understood that they are intimately mixed in the cell.[5]

[5] The following statement occurs in a recent paper by Folger (1926, p. 368). "*Mast ('07) was among the first to postulate a reversible photochemical process to account for photic response in lower organisms. Hecht ('19) elaborated this idea to explain the response to light in Mya arenaria.*" In order to make clear the developmental sequence of the ideas here involved, the complete statement of Mast's hypothesis, taken from the paper referred to (Mast, 1907, p. 159), is given verbatim. "*To explain reversal in the sense of reaction on the basis of chemical reactions induced by light let us assume: (1) That Volvox contains substances X and Y,*

The primary reaction, here written as a completely reversible reaction

$$\underset{\text{"dark"}}{\overset{\text{light}}{S \rightleftharpoons P + A}} \tag{13}$$

may indeed by only a pseudoreversible reaction

$$\begin{array}{l} \overset{\text{light}}{S \rightarrow P + A + B} \\ S \leftarrow P + A + C \\ \text{"dark"} \end{array} \tag{14}$$

in which B is the substance that actually controls the reaction $L \rightarrow T$, and C is a substance present in excess. In picturing concretely the behavior of such a system as given in the above rectangular diagram the pseudoreversible reaction is often more helpful than the simpler, completely reversible one. Mathematically, the two are identical.

Such a coupled system of two reactions describes most of the features exhibited by the photosensory process, not merely qualitatively but in quantitative detail as well. It accounts for the composition of the reaction time; for the different effects of temperature on the two parts of the reaction time; and for the interrelation of the two parts of the reaction time. It accounts for the spectral sensitivity of the animals; for the kinetics of dark adaptation, as we have seen; and for the effect of temperature on dark adaptation. It accounts for light adaptation and sensory equilibrium; for the sensitivity changes at different light levels; and for intensity discrimination. It even includes the proof of the major assumption made in the theoretical treatment of dark adaptation, namely that under all conditions of adaptation a constant photochemical effect is necessary

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chemical reaction between which is regulated by the intensity of light; (2) that a suboptimum intensity favors the formation of substances represented by X and a supraoptimum intensity those represented by Y; and (3) that the colonies are neutral in reaction when there are Y substances in one member of the equation and X in the other; positive when one member contains (X+) substances and the other (Y−), and negative when one contains (X−) and the other (Y+)." Probably the first use of the idea of a reversible photochemical reaction in relation to the photosensory process was made by Müller (1896).

to produce a definite response in the animal. The general idea involved therefore leads to a consistent and rational view of the nature of the photosensory process.

VII.

SUMMARY.

1. Data are presented for the dark adaptation of four species of animals. They show that during dark adaptation the reaction time of an animal to light of constant intensity decreases at first rapidly, then slowly, until it reaches a constant minimum.

2. On the assumption that at all stages of adaptation a given response to light involves a constant photochemical effect, it is possible to describe the progress of dark adaptation by the equation of a bimolecular reaction. This supposes, therefore, that dark adaptation represents the accumulation within the sense cells of a photosensitive material formed by the chemical combination of two other substances.

3. The chemical nature of the process is further borne out by the fact that the speed of dark adaptation is affected by the temperature. The velocity constant of the bimolecular process describing dark adaptation bears in *Mya* a relation to the temperature such that the Arrhenius equation expresses it with considerable exactness when $\mu = 17{,}400$.

4. A chemical mechanism is suggested which can account not only for the data of dark adaptation here presented, but for many other properties of the photosensory process which have already been investigated in these animals. This assumes the existence of a coupled photochemical reaction of which the secondary, "dark" reaction is catalyzed by the products of the primary photochemical reaction proper. This primary photochemical reaction itself is reversible in that its main products combine to form again the photosensitive material, whose concentration controls the behavior of the system during dark adaptation.

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HYDRATION OF GELATIN IN SOLUTION.

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The high viscosity of gelatin solutions indicates that even at temperatures as high as 40°–50°C. gelatin is hydrated,[1] *i.e.*, that the ultimate gelatin units (particles or molecules) in the solution are in some way combined with a certain amount of water. This is true not only in the case of ionized gelatin, where the hydration is regulated by the Donnan equilibrium, but also in the case of isoelectric gelatin, the viscosity of which, even at high temperatures, is quite high as compared with that of crystallized egg albumin of the same concentrations, as shown in Fig. 1. The hydration of gelatin is indicated also by measurements of osmotic pressure of various concentrations of gelatin solutions. In this respect gelatin differs from egg albumin. The curve, Fig. 2, for osmotic pressure *vs.* concentration for isoelectric albumin (sp. cond. about 8×10^{-5} in 15 per cent sol.) at 20°C. is a straight line, while in the case of gelatin the osmotic pressure increases much faster than the concentration, and the curve is convex toward the concentration axis. This difference in the behavior of gelatin and egg albumin with respect to osmotic pressure is explainable by the difference in their degree of hydration. As was shown elsewhere,[2] the osmotic pressure of dilute molal solutions of hydrated substances may be expressed as

$$P = K \frac{C}{100 - \varphi} \tag{1}$$

where $K = \frac{RT}{M}$ (M = mol weight of solute).

C = gm. of solute per 100 cc. of solution.

φ = volume of the hydrated solute,

[1] Bogue, R. H., The chemistry and technology of gelatin and glue, New York and London, 1922, 194.

[2] Kunitz, M., *J. Gen. Physiol.*, 1925–26, ix, 723.

which means that the osmotic pressure would be proportional to the concentration if the concentration were corrected for the volume occupied by the *hydrated* solute.

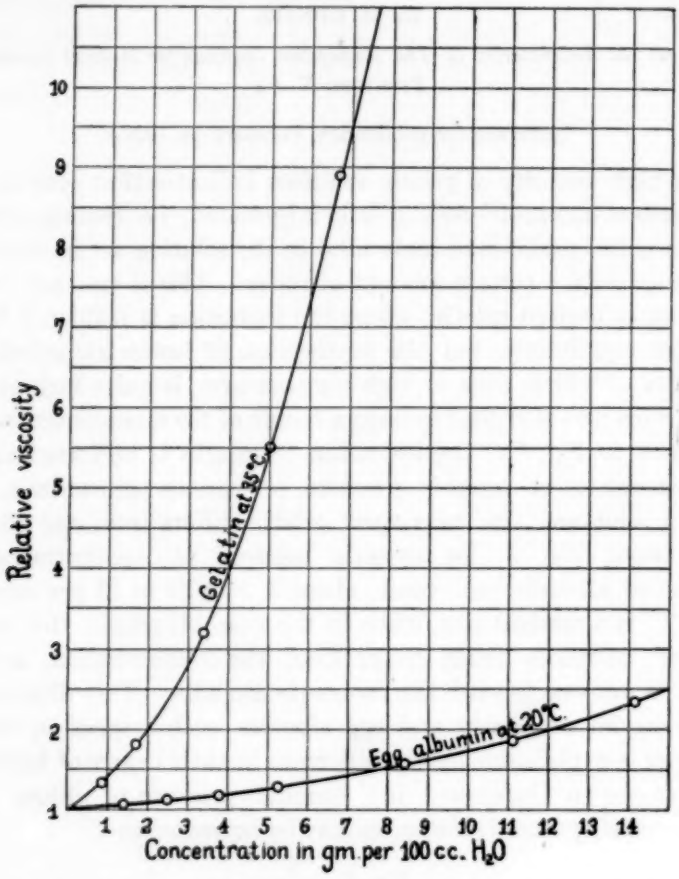

FIG. 1. Viscosity-concentration curves of isoelectric gelatin at 35°C. and of isoelectric egg albumin at 20°C.

In the case of egg albumin the correction is small, and if C is expressed in gm. per 100 cc. of H_2O, there is then practically no correction, and the plotted curve is a straight line. In the case of gelatin, on the other hand, the volume occupied by the hydrated solute is quite large, so that the active concentration of gelatin as expressed in gm.

per 100 cc. of H_2O is much greater than the one taken from dry weight measurements. Hence the osmotic pressure increases much faster than the dry weight concentration. The curves, as said before, should become straight lines if the concentrations of the gelatin solutions were corrected for the volume of the hydrated solute. A means of finding this correction is afforded by viscosity measurements.

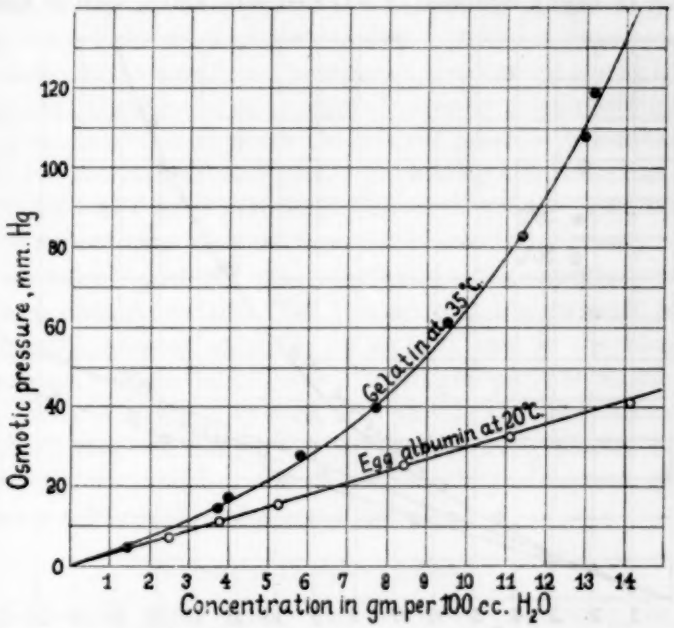

FIG. 2. Osmotic pressure-concentration curves of isoelectric gelatin at 35°C. and of isoelectric egg albumin at 20°C.

A Method for Measuring Hydration of Gelatin.

In a recent publication,[3] the writer showed that the viscosity of a number of colloidal solutions, as well as of various sugar solutions, as measured by means of an Ostwald viscosimeter, may be well represented by the equation

$$\frac{\eta}{\eta_0} = \frac{1 + 0.5\,\varphi}{(1 - \varphi)^4} \qquad (2)$$

[3] Kunitz, M., *J. Gen. Physiol.*, 1925–26, ix, 715.

where η is the absolute viscosity of the solution, η_0 is the absolute viscosity of the solvent, and φ is the volume occupied by the solute expressed as a fraction of the total volume of the solution. In the case of sugars or other substances that are hydrated to a very small extent, the value of φ when expressed as cc. per 100 cc. of solution actually equals the volume of the dissolved dry substance, while in the case of highly hydrated or solvated substances, such as casein in

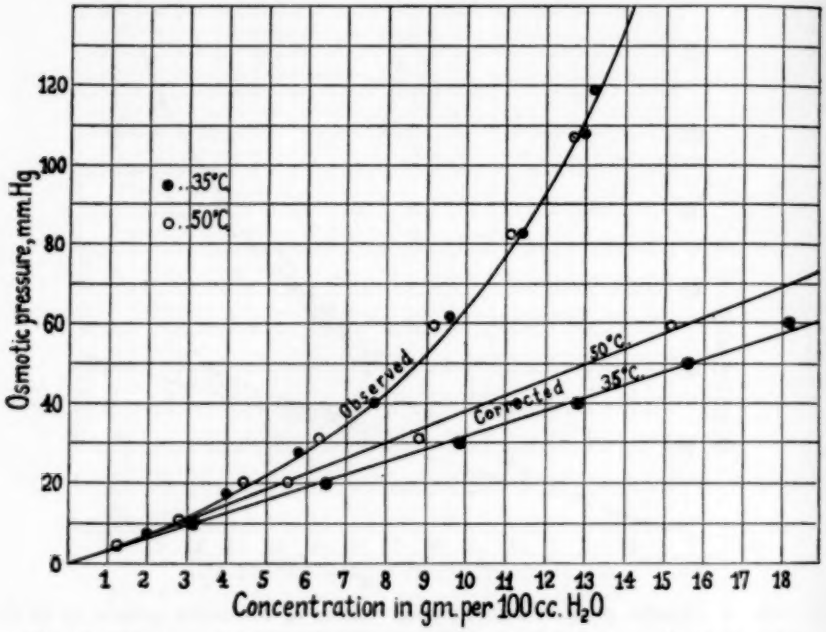

FIG. 3. Effect on the osmotic pressure-concentration curves of gelatin of correcting the concentration of the gelatin for the water of hydration as calculated from viscosity measurements.

water or rubber in benzene, φ represents the volume of the dry substance plus the volume of the solvent associated with it. This was checked for rubber by substituting the values for φ as obtained from viscosity measurements into the equation for osmotic pressure, (equation (1)). The values of K for various concentrations of rubber were constant. The data of Caspari[4] were used in both cases.

[4] Caspari, W. A., *J. Chem. Soc.*, 1914, cv, 2139.

This method of testing the validity of the viscosity formula is applied in this paper to isoelectric gelatin. A series of curves for the osmotic pressure of various concentrations of isoelectric gelatin at various temperatures were plotted. All the curves showed the characteristic convexity toward the concentration axis. The concentrations were expressed as gm. of dry gelatin per 100 cc. H_2O, as determined by drying for 24 hours at 100°C., *definite weights* of gelatin solution taken from the osmometers after equilibrium was reached. At the same time viscosity measurements were carried out by means of an Ostwald viscosimeter on samples of the same gelatin solutions at the same temperatures at which the osmotic pressure measurements were done. The viscosities of freshly prepared gelatin solutions were also measured and gave practically identical results. The various values of φ were then read off from the theoretical viscosity curve shown elsewhere,[5] and the concentrations in the osmotic pressure curves were then corrected. Fig. 3 shows that the corrected points for the osmotic pressure values lie on straight lines in the range of the dry weight concentration from 1 to 10 gm. per 100 cc. of H_2O for the various temperatures used, thus proving that the values of φ as obtained from the viscosity represent the true volumes of the hydrated gelatin particles, and hence affording a method of determining the degree of hydration of gelatin.

Molecular Weight of Gelatin.

One of the difficulties usually experienced in calculating the mol weight of gelatin from osmotic pressure through the application of van't Hoff's formula

$$P = \frac{RT}{M} C \; (C = \text{gm./cc.}),$$

is the fact that P/C, as usually plotted, is not constant. But if, on the other hand, the concentration is corrected for the "water of hydration" by means of viscosity, then RT/M becomes a constant value, expressed as the slope of the corrected curve, or it can be calculated more exactly from the relation

$$\frac{RT}{M} = \frac{P \times (100 - \varphi)}{C} \tag{3}$$

[5] Kunitz, M., *J. Gen. Physiol.*, 1925–26, ix, 717.

At 35°C. the value of RT/M is 313, as shown in Table I.

Hence

$$M = \frac{RT}{313}$$

R being the gas constant equals

$$\frac{P_0 V_0}{T_0} = \frac{22.4 \times 760 \times 1000}{273} = 62{,}400 \text{ cc.mm.Hg/degree}$$

or

$$M = \frac{62{,}400 \times 308}{313} = 61{,}500.$$

TABLE I.

C	$\frac{\eta}{\eta_0}$	φ	Corrected concentration $= \frac{C}{100-\varphi}$	P	$\frac{RT}{M}$ uncorrected $= \frac{100P}{C}$	$\frac{RT}{M}$ corrected $= \frac{(100-\varphi)P}{C}$
gm./100 cc. of solution				*mm.Hg*		
1	1.43	7.75	1.08	3.5	350	324
2	2.06	15.05	2.35	7.5	375	319
3	2.96	21.80	3.84	12.0	400	312
4	4.24	27.90	5.55	17.0	425	306
5	6.00	33.40	7.50	23.0	460	307
6	8.20	38.10	9.70	29.5	492	304
7	10.85	42.18	12.1	37.5	537	310
8	13.9	45.52	14.7	47.0	588	320
Average.....						313

This value represents the weight of dry gelatin which, if dissolved in 1000 cc. of H_2O, will produce an osmotic pressure of $22.4 \times \frac{308}{273}$ atmospheres at 35°C.

The Mechanism of Hydration of Gelatin.

The experiments of Loeb[6] on viscosity of gelatin at various pH led him to the conclusion that gelatin solutions contain a number of

[6] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 2nd edition, 1924, 270.

submicroscopic particles of solid jelly which are able to take up water and swell when there is a greater osmotic pressure inside the particles than outside. On the addition of acid or alkali to a solution of isoelectric gelatin a Donnan equilibrium is established between the diffusible ions inside and outside of the particles with the result that, owing to the larger concentration of gelatin inside, there are more ions inside than outside, and the particles then increase in volume on account of the difference in osmotic pressure. According to this view, then, these submicroscopic particles of solid jelly behave with respect to the Donnan equilibrium in agreement with the Procter-Wilson[7] theory of swelling of blocks of gelatin. According to this theory a block of gelatin under the influence of a higher ion activity inside than in the surrounding medium takes up water until the difference in the osmotic pressure between the inside and the outside solution is balanced by the stress in the elastic structure of the block, which appears to obey Hooke's law, *i.e.*,

$$e = E V$$

where e is the osmotic pressure due to the difference in the activity of ions, E is the bulk modulus, and V is the increase in volume.

This theory appears to hold also for the swelling of blocks of *isoelectric* gelatin, as was shown by Northrop and the writer.[8] In the case of isoelectric gelatin, where ions are practically absent, an osmotic pressure exists in a block of solid gelatin due to the presence of a water-soluble constituent of gelatin held in a network of insoluble fibers. This was confirmed by actually isolating from gelatin, by fractional precipitation with alcohol, two fractions, one of which is soluble in cold water, does not set to a jelly, and has a low viscosity and a high osmotic pressure, and a second one which is insoluble at ordinary temperatures, sets to a jelly in very low concentration, and swells much less than ordinary gelatin. When a block of ordinary solid isoelectric gelatin is immersed in water, the water enters the gelatin which swells until the osmotic pressure of the soluble fraction of the

[7] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307. Wilson, J. A., and Wilson, W. H., *J. Am. Chem. Soc.*, 1918, xl, 886.

[8] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1926–27, x, 161.

gelatin inside of the block is equal to the elasticity pressure of the block, *i.e.*,

$$P = E \frac{V_s - V_0}{V_0}$$

where P is the osmotic pressure in the block which can be measured directly as described,[9] E is the bulk modulus of elasticity, V_s is the final volume of the block at equilibrium, and V_0 is the volume of the dry gelatin.

It will now be shown from the viscosity measurements of various concentrations of isoelectric gelatin at 35°C. that the apparent hydration of gelatin at this temperature is due to the swelling of the ultramicroscopic particles of solid jelly brought about by the same mechanism as the swelling of large blocks of gelatin,—namely, by the osmotic pressure of the *soluble* gelatin which is included in the insoluble particles of solid jelly, or micellæ as they were named by Naegeli. The particles swell until the difference between the osmotic pressure inside and outside of the particles is balanced by the elastic pressure of the particle.

Let q be amount of water in cc. held by 1 gm. of gelatin, as calculated from viscosity, *i.e.*

$q = \frac{\varphi}{C} - .75$, the last value being the volume of 1 gm. of dry gelatin.

Let also n be the number of micellæ per gm. of gelatin.

$\frac{q}{n}$ = cc. H_2O per micella, under the assumption that all the water of hydration is associated with the micellæ only.

s = gm. of soluble gelatin per micella.

K = osmotic pressure constant for soluble gelatin, *i.e.*,

$$K = \frac{RT}{M}$$

Then the osmotic pressure inside at equilibrium equals $\frac{Ks}{q/n} = \frac{Ksn}{q}$. The osmotic pressure outside is the osmotic pressure P as determined directly, while the elastic pressure is

$$E \frac{q/n}{v_0} = E \frac{q}{v_0 n}$$

[9] Northrop and Kunitz,[8] p. 162.

where E is the bulk modulus of the micellæ at the given temperature, and v_0 is the volume of the micella before swelling. The equilibrium condition is then

$$\frac{Ksn}{q} - P - E\frac{q}{v_0 n} = 0;$$

or if instead of sn the symbol α is used, where α designates the fraction of 1 gm. of gelatin which is found in a soluble state inside of the micellæ, then the equation becomes

$$\frac{K\alpha}{q} - P - E_1 q = 0, \tag{4}$$

where

$$E_1 = \frac{E}{v_0 n}$$

As the concentration of the gelatin approaches zero, P approaches it likewise, and we have then

$$\frac{K\alpha}{q} - E_1 q = 0$$

$$[P \doteq 0]$$

or

$$\frac{K\alpha}{E_1} = q^2$$

$$[P \doteq 0]$$

If a curve is plotted for the values of q as obtained from viscosity measurements against the concentrations of gelatin used and if the curve is continued until it crosses the q axis, the value of q at the interception may then be introduced into the last equation. The actual value of q^2 as read off from the curve is close to 52. Thus we have

$$\frac{K\alpha}{E_1} = 52.$$

When P is not equal to zero the equilibrium equation becomes

$$\frac{52}{q} - \frac{P}{E_1} - q = 0 \tag{5}$$

or

$$\left(\frac{52}{q} - q\right) \div P = \frac{1}{E_1} \tag{5a}$$

The following table shows the value of $1/E_1$ for various concentrations of isoelectric gelatin at 35°C.

TABLE II.

C	P	q	$\frac{1}{E_1} = \left(\frac{52}{q} - q\right) \div P$
gm./100 cc. of solution	*mm. Hg*		
1	3.5	7.00	.123
2	7.5	6.78	.120
3	12.0	6.52	.123
4	17.0	6.30	.125
5	23.0	5.93	.123
6	29.5	5.60	.125
7	37.5	5.28	.122
8	47.0	4.94	.120
Average			.123

Substituting into equation (5) for $1/E_1$ its value of .123, and for P the relation

$$P = \frac{K_1 C}{100 - \varphi} \text{ (equation (3)), where}$$

$$K_1 = 313^* \text{ and } \varphi = (q + .75)C$$

we get

$$\frac{52}{q} - \frac{38C}{100 - (q + .75)C} - q = 0 \tag{6}$$

* Table I.

Fig. 4 shows the plotted theoretical curve for equation (6), obtained by assuming various values for q and solving for C. On the same figure are also shown the experimental values for q at various values of C, which are very close to the theoretical line. This agreement between the theoretical values of the water of hydration of gelatin with the values obtained from viscosity measurements confirms the theory that a solution of isoelectric gelatin even at 35°C. contains a definite number of small blocks of gelatin filled with a definite weight

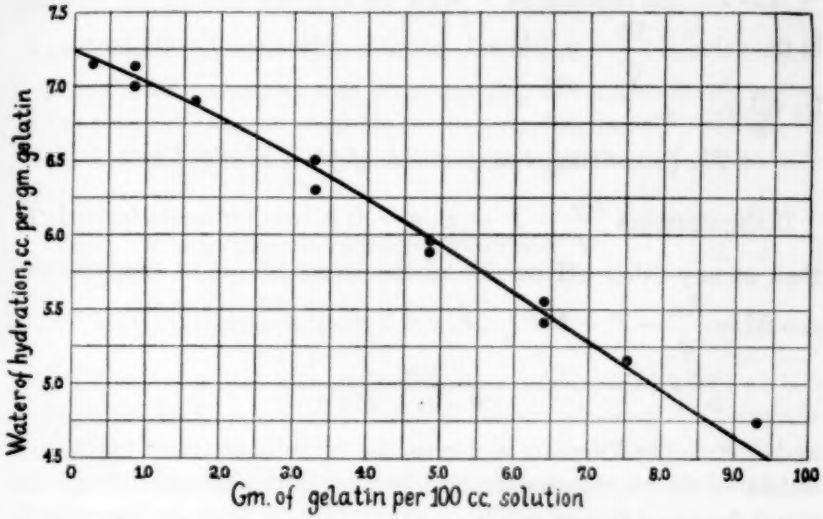

FIG. 4. Relation between the concentration of gelatin and the amount of water of hydration per gm. of dry gelatin. The dots are the values obtained from viscosity measurements. The smooth curve represents the theoretical relation:

$$\frac{52}{q} - \frac{38C}{100 - (q + .75)C} - q = 0.$$

of soluble gelatin the osmotic pressure of which is higher than the osmotic pressure of the gelatin outside of the blocks, *i.e.* higher than the total osmotic pressure of the gelatin solution as a whole. Owing to this difference in pressure, each little block swells until its osmotic pressure is balanced by the total osmotic pressure of the solution and the elastic resistance of the block to stretch.

As the concentration of the gelatin is increased the total osmotic

pressure of the solution is increased. The number of little blocks is also increased, but the amount of soluble gelatin per block is unchanged, with the result that the difference in osmotic pressure between the inside and the outside of the block continuously decreases with the increase in the total concentration of the gelatin solution. The swelling of the little blocks is thus decreasing gradually as shown on the curve. This is also clear from equation (5*a*) where $\frac{52}{q} - q = .123\ P$. An increase in P must be followed also by an increase in the value of $\frac{52}{q} - q$, which is possible only when q is diminishing in its value.

The Quantitative Interpretation of pH Viscosity Curves.

If the equation $\frac{K\alpha}{q} - P - E_1 q = 0$ is true for isoelectric gelatin then at any other pH outside of the isoelectric point the equation should be $\frac{K\alpha}{q} - P + D.P. - E_1 q = 0$ where P equals

$$\frac{313C}{100 - (q + .75)C},$$

and $D.P.$ is the difference between the osmotic pressure inside and outside of the micellæ due to the difference in the ion activity brought about by the Donnan equilibrium established between the micellæ and the gelatin solution outside of the micellæ. This will be true only if the addition of acid or alkali does not modify the values of α and E_1. That this is the case for HCl, at least, was demonstrated by the fact of the reversibility of the swelling of gelatin in an HCl solution, since on washing away the HCl the swollen gelatin returns to the original volume of isoelectric gelatin.[10] This is also true with respect to osmotic pressure. Hence, if values could be substituted for K, α, and E_1, then it would be possible to calculate the internal Donnan pressure of gelatin at various pH from the viscosity measurements. But, as found above,

$$\frac{K\alpha}{E_1} = 52$$

[10] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925–27, viii, 329.

and

$$\frac{1}{E_1} = .123 \quad \text{or} \quad E_1 = 8.1$$

Hence

$$K\alpha = 420$$

Substituting these values, the equation for the internal Donnan pressure of a solution of gelatin chloride at various pH is then

$$D.P. = P + 8.1\ q - \frac{420}{q} \tag{7}$$

There is also another way of obtaining the approximate values of the internal Donnan pressure of the micellæ; namely, through an analysis of the ion distribution between the micellæ and the outside gelatin solution.

Let H_i = hydrogen ion activity in the micellæ.
$[HCl_i]$ = total HCl concentration in the micellæ.
H_0 = hydrogen ion activity outside.
$[HCl_0]$ = total HCl concentration outside.

If it is to be assumed that the gelatin has only a slight effect on the activity of the Cl ion,[11] and that the concentration of HCl outside of the micellæ is not much different than the average concentration of HCl in the gelatin solution, then according to the Donnan equilibrium relation

$$H_i\, \gamma_{Cl_i}\, [HCl_i] = H_0\, \gamma_{Cl_0}\, [HCl_0]$$

where γ_{Cl_i} and γ_{Cl_0} are the activity coefficients for Cl ion,

or

$$H_i\, [HCl_i] = H_0\, [HCl_0]\, \frac{\gamma_{Cl_0}}{\gamma_{Cl_i}} \tag{8}$$

The calculations of Loeb[12] on osmotic pressure of gelatin have shown that the Donnan osmotic pressure of a solution of gelatin in a collodion membrane is equal approximately to the sum of the activities of the

[11] Loeb,[6] p. 50. Hitchcock, D. I., *J. Gen. Physiol.*, 1922–23, v, 387. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1924–25, vii, 34.

[12] Loeb,[6] p. 217.

ions in the gelatin solution, as measured electrometrically, multiplied by $RT/1000$. This same pressure is also acting against the Donnan pressure of the micellæ, which in its turn equals the sum of the activities of the ions in the micellæ. Hence the difference in the Donnan pressure is

$$D.P. = \frac{RT}{1000}(H_i \times Cl_i - H_0 - Cl_0) \tag{9}$$

where

$$Cl_i = \gamma_{Cl_i}[HCl_i] \text{ and } Cl_0 = \gamma_{Cl_0}[HCl_0].$$

Equation (8) may also be put in the form of

$$H_i\left(\frac{H_i}{\gamma_{H_i}} + [HCl_c]\,C\right) = \frac{\gamma_{Cl_0}}{\gamma_{Cl_i}}\,a \tag{10}$$

where $[HCl_c]$ = equivalent concentration of HCl combined with 1 gm. of gelatin when dissolved in 100 cc. of solvent, C = concentration of gelatin in gm. per 100 cc. H_2O, and $a = H_0[HCl_0]$

or
$$H_i^2 + H_i\,[HCl_c]\,\gamma_{H_i}\,C = \gamma_{H_i}\frac{\gamma_{Cl_0}}{\gamma_{Cl_i}}\,a \tag{10a}$$

If it is to be assumed, as a first approximation, that

$$\frac{\gamma_{Cl_0}}{\gamma_{Cl_i}} = 1$$

and that
$$\gamma_{H_i} = \gamma_{H_0}$$

then
$$H_i^2 + H_i\,[HCl_c]\,\gamma_{H_0}C = \gamma_{H_0}\,a \tag{10b}$$

or
$$H_i^2 + H_i\,[HCl_c]\,C_1 = a_1$$

where
$$C_1 = \gamma_{H_0}\,C \text{ and } a_1 = \gamma_{H_0}\,a$$

It will be shown later that of every gm. of gelatin dissolved at 35°C., 0.48 gm. is found in the micellæ. Hence if q is the amount of H_2O of hydration per gm. of gelatin, as obtained from viscosity measurement, the concentration of the gelatin in the micellæ, is $C = \frac{.48}{q}$, since the water of hydration is associated, according to theory developed here, with the micellæ only.

At any pH the amount of HCl combined per gm. of gelatin in the micellæ can be obtained from the titration curve of gelatin, since it was shown by experiment that there is practically no difference in the titration curves of the soluble and the insoluble fractions of gelatin. Fig. 5 shows the titration curve for 1 per cent solution of gelatin with HCl. The curve was corrected, from the data in Table IV, for the free HCl, *i.e.* for the HCl required to bring H_2O to the corresponding

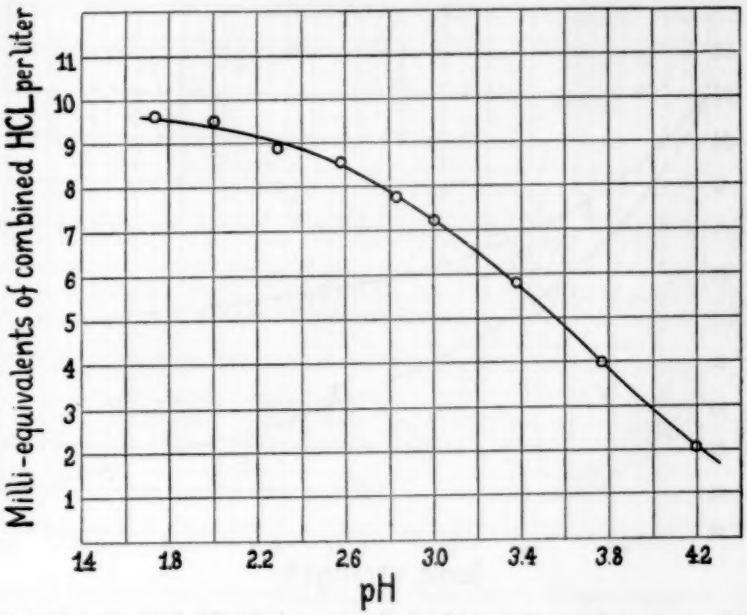

FIG. 5. Corrected titration curve of 1 per cent isoelectric gelatin with HCl.

pH. At any value of H, the value of HCl_c is thus determinable by reading it off the curve. The correction for the free HCl was based on the relation $[HCl] = \frac{H}{\gamma_H} + [HCl_c]C$ where

[HCl] = total concentration of HCl in the gelatin solution.
H = activity of the H ion as obtained from pH measurement.
γ_H = activity coefficient of H at the ionic strength equal to [HCl].*
$[HCl_c]$ and C are the same as defined before.

* Gelatin appears to affect neither the ionic strength of a solution nor the activity coefficients of the various ions. See Northrop and Kunitz,[11] p. 29.

Values for γ_H (as well as for γ_{Cl_e}, γ_{H_i}, and γ_{Cl_i}, used in the later calculations) at the various values of [HCl] were calculated, by the method of Lewis and Randall,[13] from the recent data of Scatchard[14] on the mean activity coefficients of KCl and HCl at 25°. The curves for γ_H and γ_{Cl} at various concentrations of HCl are shown on Fig. 6. All pH measurements were done electrometrically at 35°C., and the values for pH were based on 0.100 M HCl as a standard, its pH being

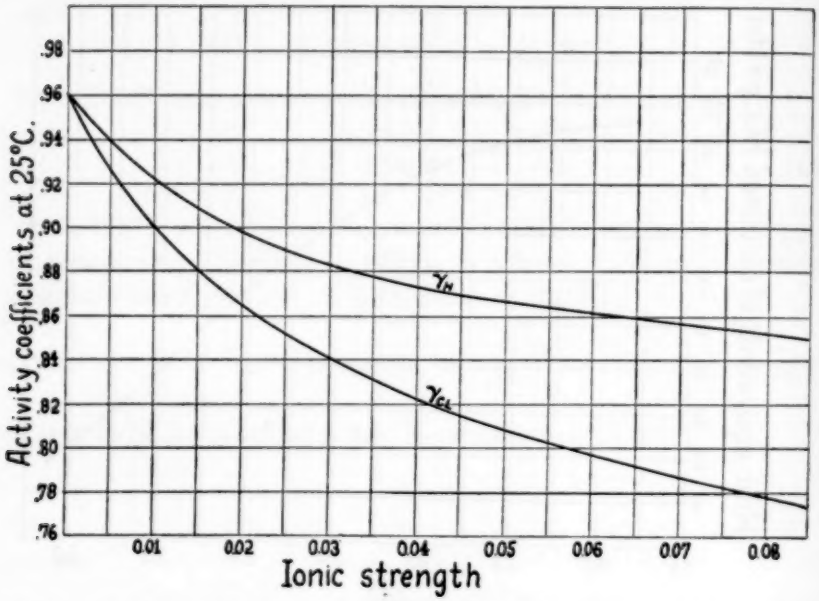

FIG. 6. Activity coefficients for hydrogen and chloride ions at various values of the ionic strength as calculated from the data of Scatchard on the mean activity coefficients of KCl and HCl at 25°C.

taken as 1.085 at 35°. It is thus possible, by means of equation (10*b*), to calculate approximately the activities of the ions in the micellæ from the known data on the whole solution and from the additional information on the concentration of the gelatin in the micellæ from the viscosity measurements, especially since $[HCl_e]$

[13] Lewis, G. N., and Randall, M., Thermodynamics and the free energy of chemical substances, New York and London, 1923, 381.

[14] Scatchard, G., *J. Am. Chem. Soc.*, 1925, xlvii, 660.

can be easily expressed as a function of H_i by means of the equilibrium equation representing the titration curve. The actual calculations were done graphically by assuming various values of H and solving for a_1 at a given value of C_1. This was repeated for the identical values of H and another value of C_1. A family of curves were then plotted for C_1 of 2.0, 2.5, 3.0, 3.5, and 4.0 of pH as abscissæ and values of a_1 as ordinates. These curves were then used for finding the pH of the

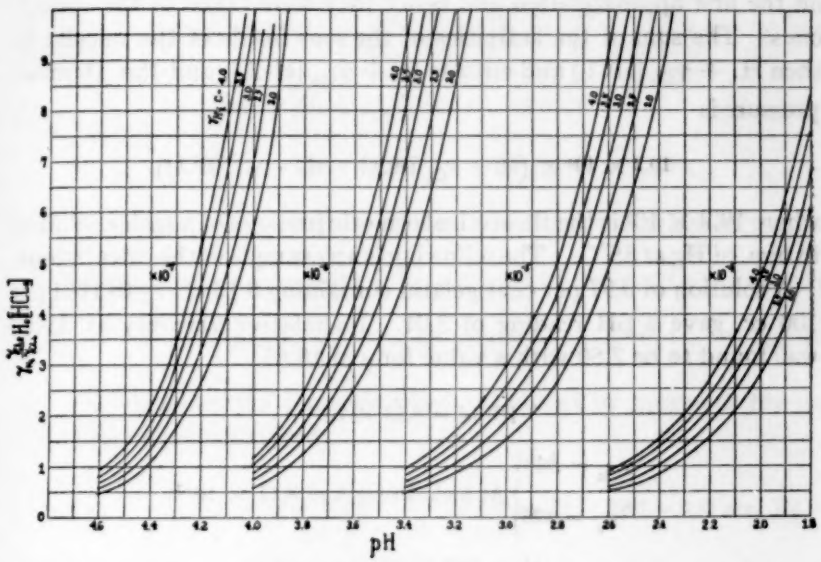

FIG. 7. Auxiliary curves for obtaining the values of pH in the micellæ of 1 per cent gelatin-HCl solution from the relation:

$$H_i^2 + H_i\,[HCl_e]\,\gamma_{H_i}\,C = \gamma_{H_i}\frac{\gamma_{Cl_0}}{\gamma_{Cl_i}}H_0\,[HCl_0]$$

micellæ for various values of $\gamma_{H_e}\,a$ and $\gamma_{H_e}C$, thus giving values of H_i. On substituting the found value of H_i in equation (8), and assuming that $\frac{\gamma_{Cl_0}}{\gamma_{Cl_i}} = 1$, the value of $[HCl_i]$ was obtained, namely $[HCl_i] = \frac{a}{H_i}$. Using the obtained approximate values of $[HCl_i]$ as the ionic strength of the solution in the micellæ, values for γ_{H_i} and γ_{Cl_i} were

read off the curves. New values of H_i and $[HCl_i]$ were then obtained in the same manner as before except instead of $a_1 = \gamma_{H_0} a$ and $C_1 = \gamma_{H_0} C$, values of $a_{,,} = \gamma_{Hi} \frac{\gamma_{Cl_0}}{\gamma_{Cl_i}} a$ and $C_{,,} = \gamma_{H_i} C$ were employed; also in equation (8) the actual values of $\frac{\gamma_{Cl_0}}{\gamma_{Cl_i}}$ were used. The new values of H_i and $[HCl_i]$ happened to be almost identical with those obtained on the first approximation and hence they were taken as the correct ones. The sum of the activities of the ions inside of the micellæ is then $H_i + \gamma_{Cl_i}[HCl_i]$ and outside $H_0 + \gamma_{Cl_0}[HCl_0]$, and the Donnan pressure is

$$19.2 \times 10^3 \times (H_i + \gamma_{Cl_i}[HCl_i] - H_0 - \gamma_{Cl_0}[HCl_0])$$

where 19.2×10^3 is the theoretical osmotic pressure of a molar solution in mm. of Hg at 35°C. The following is an example of the calculations.

A solution of 0.97 per cent gelatin containing 8.13 cc. N/10 HCl per 100 cc. gave a pH reading of 3.01. Its relative viscosity at 35°C. was found to be 2.50 with a value for q of 18.65.

$$[HCl_0] = 8.13 \times 10^{-3}$$

$$\left.\begin{aligned} \gamma_{Cl_0} &= .910 \\ \gamma_{H_0} &= .928 \end{aligned}\right\} \text{At ionic strength of } 8.13 \times 10^{-3}$$

$$H_0 = 9.77 \times 10^{-4} \text{ from pH}$$

$$a = H_0[HCl_0] = 7.94 \times 10^{-6} \text{ and } \gamma_{H_0} a = 7.36 \times 10^{-6} = a_1$$

$$C = \frac{.48}{18.65} = 2.57 \text{ gm./100 cc. } H_2O \text{ and } \gamma_{H_0} C = 2.39 = C_1$$

pH inside (from curve for $C_1 = 2.5$ and extrapolated for 2.39) = 3.31

$$H_i \text{ from } pH_i = 4.90 \times 10^{-4}$$

$$[HCl_i] = \frac{a}{H_i} = \frac{7.94 \times 10^{-6}}{4.79 \times 10^{-4}} = 16.2 \times 10^{-3}$$

$\gamma_{H_i} = .906$ and $\gamma_{Cl_i} = .878$ for the ionic strength of 16.2×10^{-3}

$$a_e = \gamma_{H_i} \frac{\gamma_{Cl_0}}{\gamma_{Cl_i}} a = .906 \times \frac{.911}{.878} \times 7.94 \times 10^{-4} = 7.46 \times 10^{-4}$$

$$C_e = \gamma_{H_i} C = .906 \times 2.57 = 2.33 \text{ gm./100 cc. } H_2O$$

Corrected $pH_i = 3.30$ (from curve for $C_e = 2.5$ and extrapolated for 2.33)

Corrected $H_i = 5.01 \times 10^{-4}$

$$\text{Corrected } [HCl_i] = \frac{\gamma_{Cl_0}}{\gamma_{Cl_i}} \times \frac{a}{H_i} = \frac{.911}{.878} \times \frac{7.94 \times 10^{-4}}{5.01 \times 10^{-4}} = 16.4 \times 10^{-4}$$

$$\text{Corrected } Cl_i = \gamma_{Cl_i} [HCl_i] = 14.4 \times 10^{-3}$$

$$Cl_0 = \gamma_{Cl_0} [HCl_0] = 7.4 \times 10^{-3}$$

Total activity of ions inside $= H_i + Cl_i = 5.01 \times 10^{-4} + 14.4 \times 10^{-3} = 14.9 \times 10^{-3}$

Total activity of ions outside $= H_0 + Cl_0 = 9.8 \times 10^{-4} + 7.4 \times 10^{-3} = 8.4 \times 10^{-3}$

Donnan pressure $= 19.2 \times 10^3 \times (14.9 - 8.4) \times 10^{-3} = 125$ mm. Hg

This Donnan pressure when calculated by means of the pressure-elasticity equation is

$$D.P. = P + 8.1\, q - \frac{420}{q} = 3.5 + 151 - 22.5 = 132 \text{ mm. Hg}$$

which is identical, within the limits of error, with the value of 125 as calculated above.

Table III shows the values of the difference in Donnan pressure in mm. of Hg between inside and outside of the micellæ in 0.97 per cent gelatin solution of various pH as calculated by both methods. The same is shown on Fig. 8. These results confirm quantitatively the theory of viscosity of gelatin of various pH, as developed by Loeb, namely, that the viscosity is regulated by osmotic forces due to the Donnan equilibrium. A difference in the activities of the ions inside and outside of the micellæ is established because the concentration of the gelatin in the micellæ is greater than the outside concentration of the gelatin in the solution.

TABLE III.

The Donnan Osmotic Pressure in Micellæ of 0.97 Per Cent

Equivalent concentration of HCl	0	2.03×10^{-3}	4.06×10^{-3}	6.10×10^{-3}
pH_0	4.8	4.20	3.76	3.38
Relative viscosity	1.40	1.62	1.99	2.32
q	6.65	9.85	14.05	17.25
Concentration of gelatin in the micellæ $= \frac{.48}{q}$	7.22	4.87	3.41	2.78
H_0 from pH		6.3×10^{-5}	1.74×10^{-4}	4.17×10^{-4}
$Cl_0 = \gamma_{Cl_0}[HCl_0]$		1.9×10^{-3}	3.8×10^{-3}	5.6×10^{-3}
H_i		2.75×10^{-5}	8.5×10^{-5}	2.09×10^{-4}
$Cl_i = \gamma_{Cl_i}[HCl_i]$		4.4×10^{-3}	7.7×10^{-3}	11.2×10^{-3}
Donnan pressure mm.Hg $19.2 \times 10^3 \times (H_i + Cl_i - H_0 - Cl_0)$		42.0	73.0	104
Donnan pressure from relation $K\alpha/q - P + D.P. - E_1 q = 0$ $P = 3.5$, $K\alpha = 420$, $E_1 = 8.1$ mm.Hg		41.0	88.0	120

III.

0.97 Per Cent Gelatin-HCl Solution at 35°C.

8.13×10^{-3}	9.14×10^{-3}	11.2×10^{-3}	14.2×10^{-3}	20.3×10^{-3}	30.5×10^{-3}
3.01	2.83	2.58	2.29	2.01	1.73
2.50	2.54	2.52	2.40	2.19	1.99
18.65	18.95	18.85	18.75	16.05	14.10
2.57	2.53	2.54	2.56	2.99	3.40
9.77×10^{-4}	1.48×10^{-3}	2.63×10^{-3}	5.13×10^{-3}	9.8×10^{-3}	18.6×10^{-3}
7.4×10^{-3}	8.3×10^{-3}	10.0×10^{-3}	12.6×10^{-3}	17.6×10^{-3}	25.6×10^{-3}
5.01×10^{-4}	7.59×10^{-4}	1.48×10^{-3}	2.95×10^{-3}	5.98×10^{-3}	12.9×10^{-3}
14.4×10^{-3}	16.1×10^{-3}	17.8×10^{-3}	21.9×10^{-3}	28.8×10^{-3}	36.9×10^{-3}
125	134	128	138	138	107
132	136	135	133	108	88

Effect of Concentration of Gelatin on the pH—Viscosity Curves.

With the increase in the total concentration of the gelatin solution the difference in the gelatin concentration between the micellæ and the outside is gradually diminished. Hence the increase in viscosity at pH 3.0 over that of isoelectric gelatin, which as shown above is

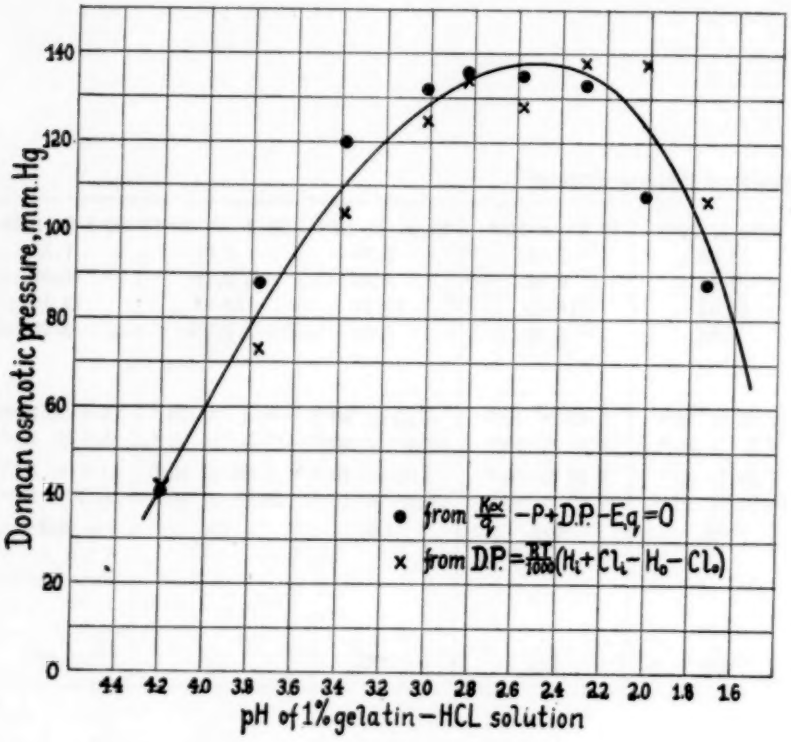
Donnan osmotic pressure, mm. Hg
140
120
100
80
60
40
20
0
4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4 2.2 2.0 1.8 1.6
pH of 1% gelatin–HCL solution
● from $\frac{K\alpha x}{q} - P + D.P. - E.q = 0$
× from $D.P. = \frac{RT}{1000}(H_i + Cl_i - H_o - Cl_o)$

FIG. 8. The difference in the Donnan osmotic pressure between the micellæ and the outside solution at various values of pH in 1 per cent gelatin-HCl solution at 35°C. as calculated from viscosity measurements.

brought about by the difference in the gelatin concentration of the micellæ, should become less conspicuous with increase in the total concentration of the gelatin solution. That this is exactly what happens is shown in Table IV.

The effect of the concentration of the gelatin solution on the

viscosity-pH curves is shown still more strikingly if instead of the relative viscosity values the values of q, *i.e.*, of the volume of H_2O taken up by a gm. of gelatin as calculated from equation (2), are used in plotting the curves. This is shown on Fig. 9. The enormous effect of the concentration of the gelatin on the viscosity that is caused by the Donnan equilibrium between the micellæ and the outside solution is apparent.

The curves show that at a concentration of 10 gm. of dry gelatin per 100 cc. of H_2O the Donnan effect on the viscosity of the solution disappears entirely. This indicates that at this concentration of gelatin there is no difference between the concentration of the gelatin inside and outside of the micellæ. Let α_1 be the fraction of each gm.

TABLE IV.

Viscosity Measurement of Various Concentrations of Gelatin, pH 4.7 and pH 3.0, at 37°C.

Concentration in gm. per 100 cc. solution	0.5	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
Relative viscosity of gelatin, pH 4.7	1.16	1.43	1.95	2.75	3.83	5.28	6.70	12.4	21.3
Additional viscosity = relative viscosity − 1	0.16	0.43	0.95	1.75	2.83	4.28	5.70	11.4	20.3
Relative viscosity of gelatin, pH 3.0	1.84	2.39	3.44	4.54	5.78	7.12	9.06	14.2	22.0
Additional viscosity	0.84	1.39	2.44	3.54	4.78	6.12	8.06	13.2	21.0
Ratio of additional viscosity, pH 3.0/pH 4.7	5.24	3.23	2.57	2.02	1.69	1.43	1.42	1.16	1.03

of dry gelatin found in the micellæ, then the concentration of the gelatin inside of the micellæ is α_1/q since q is the volume of H_2O containing α_1 gm. of gelatin. Hence in a solution of gelatin containing 10 gm. of dry gelatin per 100 cc. of H_2O

$$\frac{\alpha_1}{q} = \frac{10}{100}$$

Substituting the value of q, we get

$$\frac{\alpha_1}{4.8} = \frac{10}{100}$$

or

$$\alpha_1 = 0.48$$

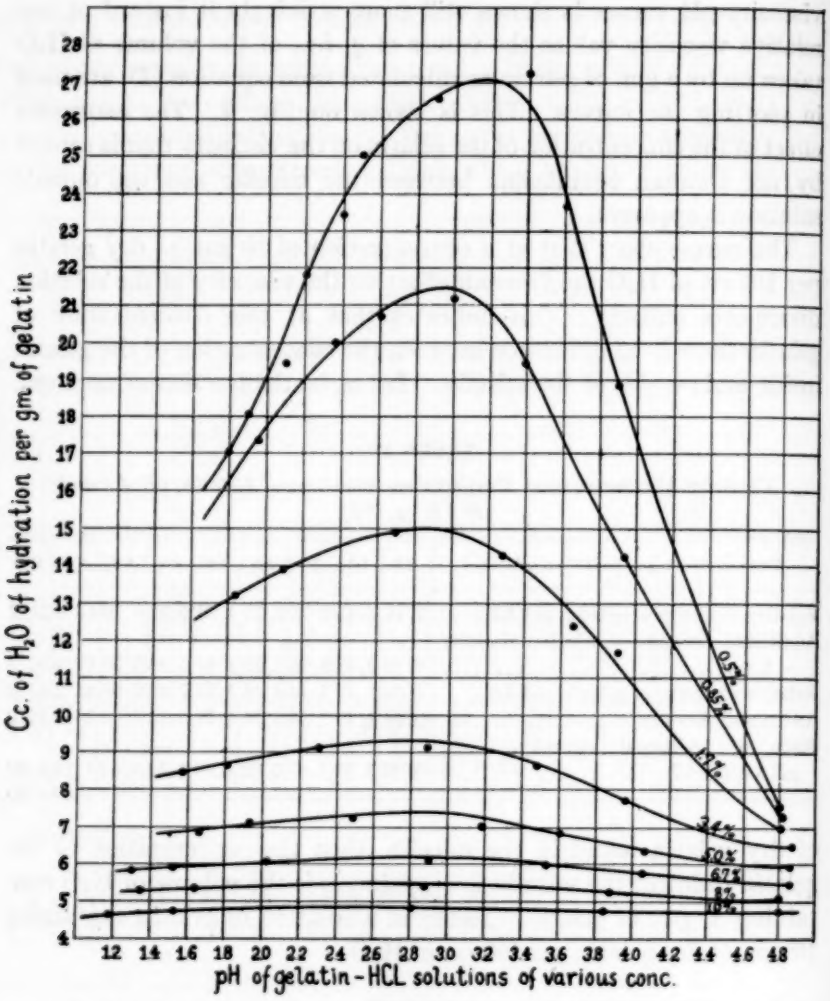

FIG. 9. Effect of concentration of gelatin on the pH-viscosity curves of gelatin-HCl solutions. The viscosity values are expressed in cc. of H_2O of hydration per gm. of gelatin.

This value of α_1 has been used in calculating the concentration of the gelatin in the micellæ of a 1 per cent solution of gelatin at various pH on the assumption that the relation between the fractions of the soluble and insoluble components of gelatin is not affected, within

certain limits, by the total concentration of the gelatin or by the pH of the solution. The agreement between the values for the Donnan pressure in the micellæ as calculated by two independent methods shows that the assumption is correct.

SUMMARY.

1. It was shown that the high viscosity of gelatin solutions as well as the character of the osmotic pressure-concentration curves indicates that gelatin is hydrated even at temperatures as high as 50°C.

2. The degree of hydration of gelatin was determined by means of viscosity measurements through the application of the formula $\frac{\eta}{\eta_0} = \frac{1 + 0.5\,\varphi}{(1 - \varphi)^4}$.

3. When the concentration of gelatin was corrected for the volume of water of hydration as obtained from the viscosity measurements, the relation between the osmotic pressure of various concentrations of gelatin and the corrected concentrations became linear, thus making it possible to determine the apparent molecular weight of gelatin through the application of van't Hoff's law. The molecular weight of gelatin at 35°C. proved to be 61,500.

4. A study was made of the mechanism of hydration of gelatin and it was shown that the experimental data agree with the theory that the hydration of gelatin is a pure osmotic pressure phenomenon brought about by the presence in gelatin of a number of insoluble micellæ containing a definite amount of a soluble ingredient of gelatin. As long as there is a difference in the osmotic pressure between the inside of the micellæ and the outside gelatin solution the micellæ swell until an equilibrium is established at which the osmotic pressure inside of the micellæ is balanced by the total osmotic pressure of the gelatin solution and by the elasticity pressure of the micellæ.

5. On addition of HCl to isoelectric gelatin the total activity of ions inside of the micellæ is greater than in the outside solution due to a greater concentration of protein in the micellæ. This brings about a further swelling of the micellæ until a Donnan equilibrium is established in the ion distribution accompanied by an equilibrium in the osmotic pressure. Through the application of the theory developed here it was possible actually to calculate the osmotic pressure difference

between the inside of the micellæ and the outside solution which was brought about by the difference in the ion distribution.

6. According to the same theory the effect of pH on viscosity of gelatin should diminish with increase in concentration of gelatin, since the difference in the concentration of the protein inside and outside of the micellæ also decreases. This was confirmed experimentally. At concentrations above 8 gm. per 100 gm. of H_2O there is very little difference in the viscosity of gelatin of various pH as compared with that of isoelectric gelatin.

The writer wishes to acknowledge his indebtedness to Dr. J. H. Northrop for valuable advice and suggestions.

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